Federation Proceedings

VOLUME 14 *September 1955* NUMBER 3

JOINT SESSION OF THE FEDERATION

San Francisco, California, April 12, 1955

Integration of Cell Structure and Function

Chairman: D. MURRAY ANGEVINE

THE FINE STRUCTURE OF CELLS

KEITH R. PORTER

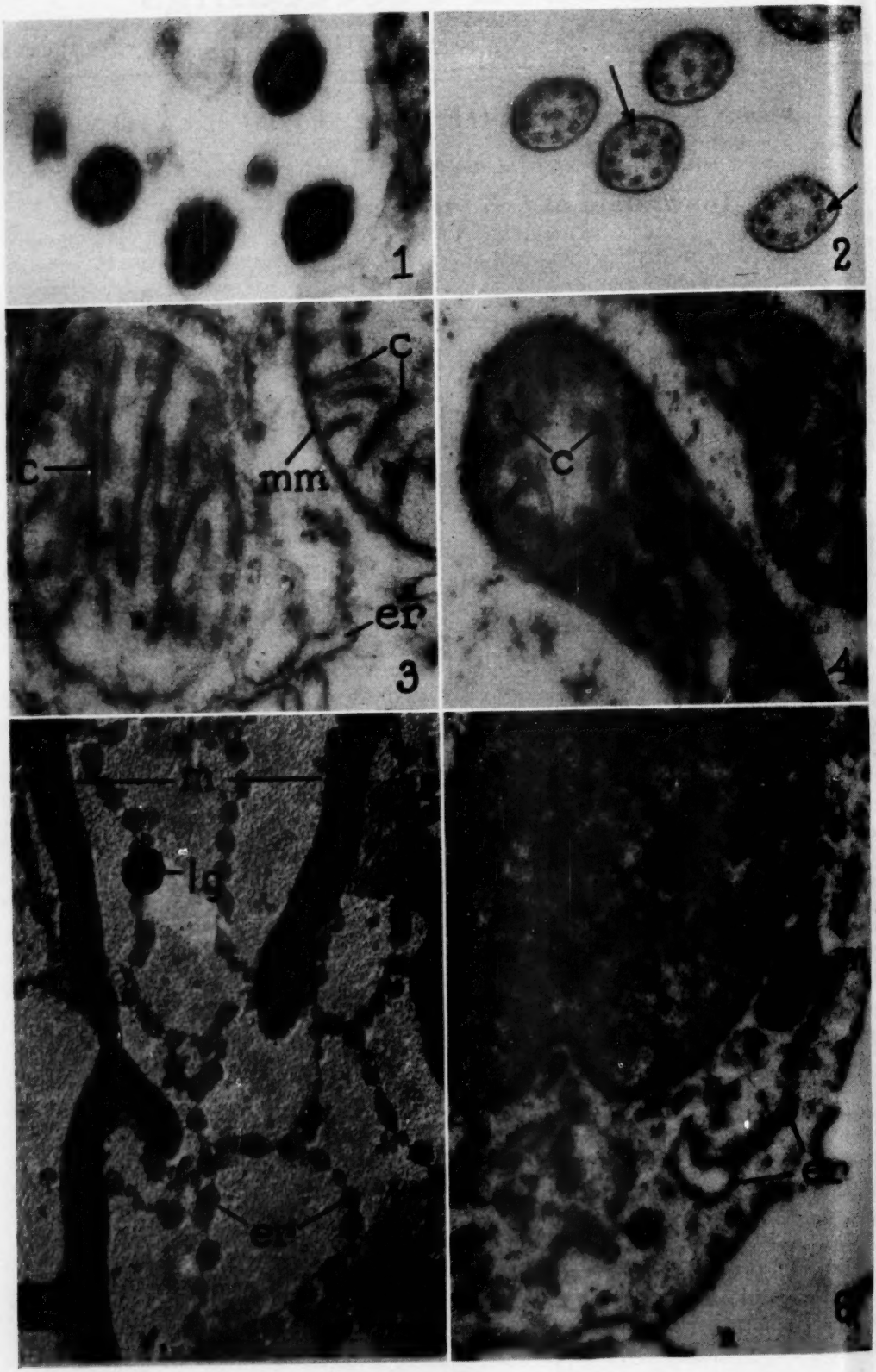
From the Rockefeller Institute for Medical Research, New York City

ELECTRON microscopy of cells, though a fairly recent pursuit of biologists, has already provided a great wealth of new information, far in excess of what can be included in a brief talk. It is planned, therefore, to confine the discussion to a few of the more recently described elements of cell fine structure which have been the special interest of our laboratory and which seem most appropriate for this program.

The parade of micrographs of cell fine structure which is currently appearing in the literature is conceivably perplexing for many readers of biological literature. Micrographs at enormous magnifications of 50–100 thousand diameters depict fibrils, particles, double membranes, vesicles, reticula and even more unusual structures with new names such as cristae and cisternae, without much regard for or reference to the old-fashioned and familiar picture of a cell. Though there is some confusion in terminology and interpretation it is probably not greater than that which usually characterizes a new field. The more surprising and encouraging thing is that even in this preliminary phase, a few general patterns of cell fine structure are emerging. These are sufficiently ubiquitous in their occurrence to be classed among the fundamental structures of the cell.

An excellent example of repetition of structural pattern is to be found in the electron microscope images of cilia (1). It will be recalled that these small protoplasmic extensions from the surface of the cell are capable of a pendular or spiral motion which either moves the immediate environment past the cell or the cell through its medium. The cilia may be long or short (1–10 μ) and occasionally occur in such large numbers as to give the cell surface in the light microscope image a pubescent appearance. The proximal or attached end of each cilium is anchored in the cell in what is usually referred to as a basal body or corpuscle, and with these there are frequently associated intracellular fibers or ciliary rootlets that are striated in a manner characteristic of protein fibers.

Electron micrographs of longitudinal sections of cilia are not especially informative as far as the lash of the cilium is concerned. They show evidence of the presence of longitudinal filaments (150–300Å in diameter) within the body of the cilium, just as several observers have noted, and also an enveloping membrane (2, 3). It is in pictures of cross sections that the more interesting attributes of cilia are revealed. The filaments, evident in longitudinal sections, are found in all to number 11; of these, 9 are evenly spaced in a ring within the cortex of the cilium, and 2 lie in the center (figs. 1 and 2). Higher resolution micrographs provide the further information that each of the peripheral filaments is double and that each element of the pair is closely similar in size to one of the central filaments. Thus it appears that each cilium is supplied with 10 pairs of filaments, 9 peripheral and 1 central. The central pair is oriented in such a way that the plane which includes the two filaments also includes


1
2
c
mm
c
er
3
c
4
lg
er
5

one of the peripheral pairs and divides the cilium into two equal halves. Where the cilium has a pendular motion, as in the gill epithelium of Mya, this plane of bilaterality is normal to the direction of beat, as would indeed be expected for mechanical reasons.

The functional significance of this fairly precise organization is obscure. It is attractive, of course, to regard the filaments as contractile elements capable of influencing the various bendings and contortions of the cilium, but that they possess this property has yet to be demonstrated. It does seem reasonable to conclude that the complex structure defined has some biological value, for throughout the evolution of higher forms the same pattern has been retained. In other words, the arrangement of filaments is precisely the same whether the cilia are found in the oviduct of the human female or on the back of a protozoan (refs. 2, 3 and figs. 1 and 2). It is, moreover, the skeletal structure of plant cilia wherever these occur, e.g. on algal zoospores and fern spermatozoids (4) and it forms the core of the single cilium or flagellum, whether as part of the mammalian sperm (5–8) or the algal spermatozoid (e.g., *Fucus*) (4).

The literature now contains accounts of several other patterns of structure which are remarkably ubiquitous in their occurrence. Among these, the internal morphology of mitochondria is a fairly striking example. The variation between mitochondria of different cell types is greater than between cilia, but the general structural attributes are similar in all cases. It is, therefore, now regarded as characteristic for mitochondria to show a number of internal lamellae (refs. 9–11 and fig. 3). These, it appears, are essentially folds directed internally from the innermost of two membranes which constitute the wall of these organelles. These folds or ridges (the 'cristae mitochondriales' of Palade) extend only part way across the cavity of the mitochondrion, so that the enclosed space within the organelle is a continuous one. They are quite uniform in thickness (usually about 200Å) but vary considerably in number. Such structures obviously provide the mitochondria with a greater area of internal surface, upon which are spread, presumably, certain of the several oxidative enzymes and enzyme chains known to be present in these organelles. It is too early to say whether there is any significant correlation between the number of cristae, i.e. the total surface provided, and the metabolic characteristics of the cell in which the mitochondria are found. But distinctions such as that between liver mitochondria, where the number of cristae is small, and heart muscle, where the number is much greater, are easily made and probably very meaningful. The present purpose is to direct attention to the fundamental nature of this internal structure which is repeated in all forms, including protozoa, though in this latter instance the cristae have the form of microvilli rather than folds (fig. 4).

FIG. 1. Electron micrograph of transverse section through four cilia of epithelial cell lining human Fallopian tube. The image shows nine peripheral (double) and two central (single) filaments characteristic of all cilia. These are embedded in a matrix material of appreciable density but without evident structure. The cilium is apparently limited by a thin membrane (× 61,000).

FIG. 2. Transverse sections through cilia of *Paramecium multimicronucleatum*. Peripheral and central filaments are characteristically arranged. The marginal nine are individually double, and each unit of the pair is similar in size and appearance to the single filaments in the center of the cilium. In cross section the filaments are defined by a dense line around a less dense center, as though possessing a tubular structure. Their outside diameter is ~ 150 Å. The membrane limiting the cilium is better resolved here than in preceding figure (× 45,000).

FIG. 3. Micrograph of thin section through portions of two mitochondria from rat liver. The double membrane surrounding the mitochondrion is evident at *mm*, internal folds or cristae are shown at *c*, and tubules of endoplasmic reticulum at *er* (× 70,000).

FIG. 4. Micrograph of mitochondria found in cortex of *Paramecium multimicronucleatum*. Edge-on view of mitochondrial membrane not apparent because plane of section cuts obliquely through organelle margin. The cristae in these mitochondria are shaped like microvilli, as indicated by outlines of cross sections, *c* among the longitudinal (× 46,000).

FIG. 5. Electron micrograph showing portion of thinly-spread macrophage from adult chick. This is an image of the entire content of the cytoplasm within this small area of the cell and not a thin section. Mitochondria, one a branching form, are indicated at *m*, lipid granule at *lg* and vesiculated strands of endoplasmic reticulum at *er* (× 17,000).

FIG. 6. Portion of thin section through chick macrophage fixed *in situ*. Beside nucleus *n*, and mitochondrion *m*, it shows profiles through vesicular elements of the endoplasmic reticulum *er*. These, it may be noted, are of approximately the same size as those shown in the cell fixed *in vitro* (fig. 5) and likewise disposed in rows or strands as evident at *er* (× 28,000).

Distinctive patterns of organization have been found to repeat in the structure of chloroplasts (12–14) and indeed in all photoreceptors (15). Certain fundamental features of structure are also evident in myofibrils wherever found (16–19), in bridges between cells (20) and in fibrous membranes outside of cells (21).

In large part, this new information about the structure of cilia and mitochondria and other components of cells and tissues is a product of technics devised only recently for cutting thin sections. The procedures involve the fixation or preservation of the cell structure in a near-to-native state with buffered solutions of OsO_4 followed by dehydration with alcohol and embedding in a plastic matrix. Sectioning is accomplished with glass knives and specially-designed microtomes. The latter instruments, of which several models are now available, have the demonstrable ability to cut serial sections as thin as 250Å with considerable regularity. Thus it is that currently cells and tissues are prepared for high resolution microscopy with as little difficulty as for light microscopy (22–24).

Not all the images of cell fine structure provided by electron microscopy of thin sections are as easy to analyze and translate into useful information as those of cilia and mitochondria. This is particularly true for some of the larger structural systems of the cell which vary considerably from one cell type to another and even between cells of identical type. In the face of such variation and the relatively minute sample provided by a usefully thin section, it is sometimes difficult to define the form of the entire structure or even small parts thereof. It is accordingly somewhat hazardous, on the basis of this type of analysis, to characterize such highly variable structures as constituting a single class or type of cell inclusion. Nonetheless to do so is important, for it not only lends order to the new information but permits one to derive from observed correlations between form and cell type a few clues to the functional significance of the system represented.

One element of cytoplasmic fine structure that is commonly-occurring and that exemplifies this variability was discovered relatively early in the application of electron microscopy to the study of cells. It may be described in a few words as a finely-divided vacuolar system of the cytoplasm. In some types of cells it constitutes a conspicuous element of the cytoplasm; in other types it is remarkably inconspicuous. Even in identical cells under identical environmental conditions it may, for no obvious reason, be either finely divided or coarsely vesicular. It has been shown in some cells to be associated with the basophilic or RNA-containing component of the cytoplasm. And there is now good evidence that it is the in situ equivalent of the microsomal fraction, as originally isolated and described by Claude (25).

The earliest images of this newly-defined component of the cell were obtained from electron microscopy of cultured cells that, in some instances, are thin enough for electron penetration. This was before adequate technics had been developed for cutting thin sections. These images described the structure simply as a lacework of greater density present in the optically-empty ground substance of the cytoplasm (26). Subsequent improvements in preparation procedures and microscopy defined the aforementioned lacework as a complex reticulum of smooth or vesiculated strands (ref. 27 and fig. 5). It was further made evident that the structure was limited by a membrane and that its content largely evaporated when the cell was dried. The concept of the network as a finely-divided vacuolar system grew out of these few observations.

Variations observed in the form of the system were striking. In some cells it appeared as a continuous system of slender canaliculi (100 mμ or less in diameter) and in others as a discontinuous or dispersed system of separate vesicles (300 mμ or more). The existence of such differences between adjacent cells of identical type in the same preparation defined the structure as extraordinarily labile and probably responsive to slight changes in the physiological state of the cell. The distribution of the system in these cultured cells placed it as a component of the endoplasmic or more generally solated portion of the cytoplasm and from this and its form it came to be referred to as the endoplasmic reticulum. Its common occurrence in all cells examined in this type of preparation suggested that it represented a ubiquitous and probably fundamental component of the cell.

The possibility was not overlooked that the structure might represent an artifact of preparation procedures, especially since it showed such extreme pleomorphism. Attempts were therefore made to see it in living cells by phase contrast and dark-field microscopy. In some instances this was successful and reasonably so because, as

pointed out above, the dimensions of the strands and vesicles observed in the electron microscope images were occasionally greater than those of mitochondria, and so well within the range of light resolution. It was noted also that a pattern of similar character and distribution could be demonstrated in some cultured cells after staining with hematoxylin or with other basic dyes, and this was accepted as evidence that the system represents or is associated with the basophilic component of the cytoplasm.

The limited usefulness of cultured cells for electron microscopy of this and other cell components is obvious. In the case of the endoplasmic reticulum, for example, the three-dimensional form of the subunits of the system was destroyed by the flattening inherent in drying. Internal structure, if any, in the units of the system was concealed by the membranes limiting the system. It was thought, moreover, that the form and distribution of the system depicted to good advantage in these thinly-spread cells might depart appreciably from that present in the equivalent cell examined *in situ*. But most limiting of all was the fact that only a small variety of cells could be cultured in a form really useful for microscopy and almost none in their native differentiated form. For reasons of this nature the demonstrable usefulness of cultured cells did not greatly reduce the need for adequately thin sections, especially for investigations of this complex vacuolar system.

One of the first interests in examining sections of cells and tissues fixed *in situ* was to see whether this newly-defined component of the cytoplasm could be identified and how it would appear in cells of the same type as those studied after *in vitro* cultivation (figs. 5 and 6). Several comparisons were eventually made and, in each case, little or no difficulty was encountered in finding profiles of vesicles or tubules in thin sections of cells of the intact tissue (28). Such profiles were easily identified by size and distribution as representing sections through trabeculae of the endoplasmic reticulum (fig. 6). A special differentiated variant of the system having the form of large, flattened vesicles (called cisternae) was defined by the thin sections and thereafter recognized in the preparations of whole cultured cells. The tridimensional reticular character of the system in cells *in situ* was demonstrated with serial sections (23, 28). It is important to note, however, that while evidence from sectioned material substantiated the observations made on cultured cells it did not entirely replace them, for it was the early pictures of cells *in toto* which defined the system as a reticulum, a fact that would have been tardily recognized from thin sections alone.

Subsequent studies using sectioned material have sought to determine whether this vacuolar system is a generally-occurring one and, if so, in what manner it varies in form and distribution among different types of cells. It may be assumed, as mentioned earlier, that from the variations in form and relation to other cell components some indication of function may become evident. A wide variety of cells has now been studied by these procedures. In all cases except mature erythrocytes, the cytoplasmic ground substance has been found to contain membrane-bound vesicles or tubules of the same general character as those encountered in the cultured cells. In certain types of cells they have been observed to adopt a distinctive form and organization which could reasonably be interpreted as expressions of cell differentiation in the usual sense.

In striated muscle cells, for example, the reticulum is present as a delicate lace-like sleeve around and between the myofibrils (fig. 7). In liver, it frequently appears as slender canalicular elements which are loosely bundled together into skeins (fig. 8). In other types of cells (e.g., epidermal or spermatocytes) the vesicular elements may be completely independent from one another, forming a discontinuous vacuolar system. These descriptive comments refer to the reticulum as it occurs in the cytoplasm outside the immediate vicinity of the centrosome where the fine structure is characteristically different.

The form adopted by the system in cells producing protein-rich secretions (e.g., acinar cells of parotid or pancreas) is especially characteristic. Here the profiles of the trabeculae of the reticulum are generally not round but very elongate (fig. 9). They vary somewhat in width (50–150 mμ) and greatly in length. At first glance they might be regarded as representing longitudinal sections through tubules. It is evident, however, from the continuity of well-defined membranes around the ends of the majority of the profiles, from the frequency of their appearance in these cells (highly improbable statistically if tubules), and most especially from a study of serial sections, that the profiles represent sections

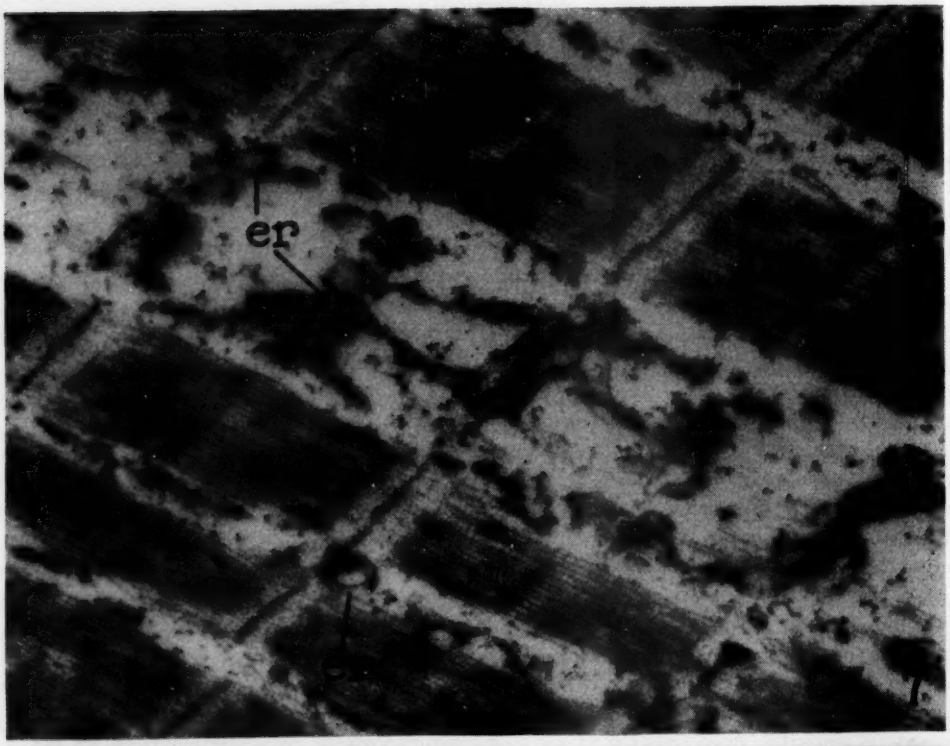


FIG. 7. Micrograph of thin longitudinal section of striated muscle from myotome of tail of *Amblystoma* larva. Membranous elements, vesicles and canaliculi of the endoplasmic reticulum are shown in the sarcoplasm between the myofibrils, *mf* (× 30,000).

FIG. 8. Thin section through portion of rat liver cell. Margin of cell nucleus (*n*) is in field at left, sectioned mitochondria at *m* and slender canaliculi in longitudinal section at *er* (× 33,000).

through thin lamellar vesicles or cisternae. These show varying degrees of fenestration (constituting reticular sheets) with different physiological conditions of the cell.

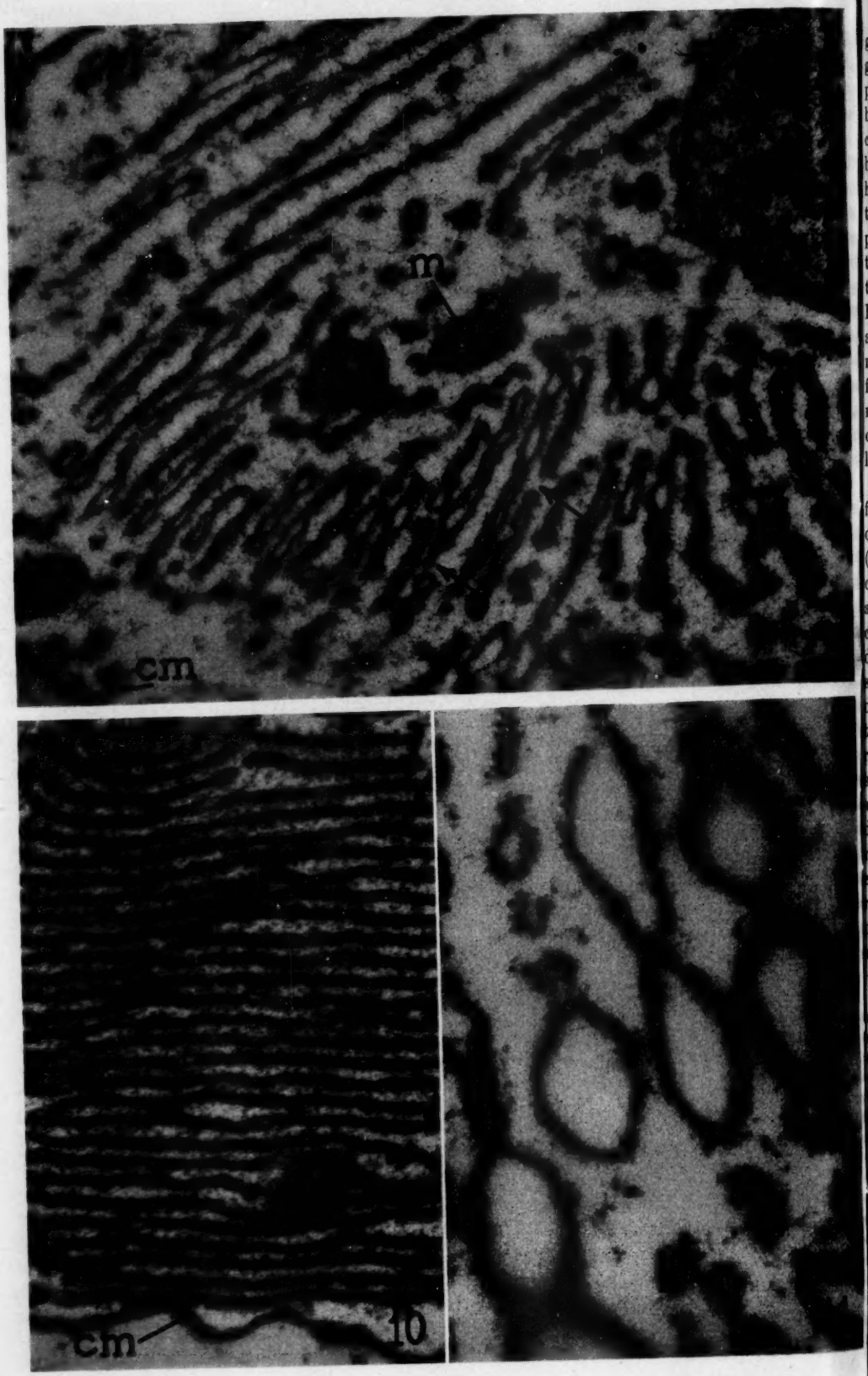
The system is obviously designed to provide these cells with as large an internal area of membrane surface as possible, but there is doubtless more purpose in the plan than this implies. The content of the vesicles, for example, is separated from the cytoplasmic matrix by the membrane which presumably influences diffusion between vesicles and matrix. There is no evidence of structure within the vesicles (figs. 9 and 11). However, the thickness of these flattened elements is noted as remarkably uniform (~50 mμ) under conditions regarded as normal, and perhaps more remarkable still is the tendency shown by the vesicles to retain a parallel orientation (figs. 9 and 10). This is maintained without the involvement of resolvable ordered structure in the intervening spaces. Possibly the fixation has not provided for a faithful preservation of order if present, but it seems more likely that the forces involved operate through polarization of the highly hydrated elements of the intervening matrix.

The striking lability of the endoplasmic reticulum noted earlier in cultured cells is easily demonstrated as characteristic of this more highly differentiated form of the system. Frequently in isolating the tissue sample for fixation slight damage will be inflicted on the marginal cells of the block, and in such cells it is common to find the flattened cisternae undergoing various degrees of vesiculation (figs. 9 and 11). In the ultimate expression of this the continuous and expansive flat cisterna is transformed into a layer of independent vesicles, which in their arrangement may retain a semblance of the orientation of the original cisterna (fig. 9). This is probably a state of degradation which is reversible. The same may not be true of changes in the system induced by slight alterations in the tonicity of the medium. When, for example, a small piece of parotid tissue is immersed in hypotonic Tyrode (0.6 NaCl instead of 0.85) for 10 minutes before fixation, the well ordered layering of the vesicles disappears entirely and is replaced by much larger sinuses of irregular outline and continuity. Thus the system, as a special differentiation of these exocrine cells, is extraordinarily unstable, resembling in this as in other characteristics the reticulum of the cultured cells.

Besides showing a preferred orientation with respect to one another, these flattened elements of the reticulum in glandular epithelium also occupy a special and preferred location within the cell, especially where the cell has formed its normal complement of secretory granules. In such cases, the cisternae adopt a parallel arrangement along the basal margins of the cell and so coincide in distribution with the basophilic component of the cytoplasm. This fact has been recognized by several workers (29–31). It has received further support from observations on experimental or functional dislocations of the basophilic component, in which case the cisternae were observed to move in parallel with the affinity for basic dyes (32, 33). In this type of cell, represented especially by the acinar cells of the pancreas and parotid, there is, therefore, a close correlation between the special aggregations of these cisternae (a special form of the endoplasmic reticulum) and the basophilic component of the cytoplasm—the ergastoplasm of Garnier (34). The same correlation is evident from the study of basophilic Nissl bodies (35) or the intensely basophilic cytoplasm of plasma cells (36).

The significance of this correlation is obvious. It indicates that the cytoplasmic RNA, which is responsible for basophilia, is in some way associated with these structures and that the system probably functions in the protein synthesis characteristic of these particular types of cells. The problem was to determine the nature of this RNA association, or where the RNA is located with respect to these elements. Is it contained within the vesicles, is it part of the membrane or is it part of the cytoplasmic matrix between these flattened vesicles? The answers to these questions have come from some recent studies of Palade (36).

In the first place, it is perfectly clear at higher magnifications that there is more in these intensely basophilic regions of acinar cells than the membrane-bound cisternae of the endoplasmic reticulum or ergastoplasm. Micrographs of such regions show a fairly dense accumulation of small dense particles (100–150Å in diameter) in between the cisternae (figs. 9 and 11). The majority of these are closely applied to the membrane surfaces, but a certain proportion are always free in the cytoplasmic matrix. The distribution of particles of this general character among various types of cells has now been carefully studied (36) and found to be fairly widespread. They are always especially concentrated around the cisternae of the endoplasmic reticulum in the re-


m
cm
cm
10

gions of intense basophilia characteristic of many gland cells. They are also abundant in the cytoplasmic matrix of rapidly-growing cells of embryonic or regenerating tissues. In these latter cells, which are diffusely basophilic, the membrane-bound elements of the reticulum are, however, far from prominent. Furthermore, in cytoplasms which show no basophilia and are acidophilic, such as granulocytes and spermatocytes, there are usually few or no granules, yet sometimes a prominent development of vesicles. Hence the separation of particles and reticulum and a clear-cut indication that the particles and not the vacuolar elements are rich in RNA. As further evidence of this association it may be noted that nucleoli have been observed that are composed almost solely of small dense particulates of approximately this same size—150Å (32).

It is appropriate now to turn to studies of cell fractions to see where these have located the bulk of the cytoplasmic RNA. Early papers of Claude (25) and others (37) reporting analyses of the so-called microsome fraction, describe the fraction as containing as much as 50% of the entire RNA content of the rat and mouse liver cell. The evidence then available defined the fraction as consisting of submicroscopic elements 80–150 mμ in diameter, and the suggestion was later made that these elements were derived from the endoplasmic reticulum (27). Simultaneous and subsequent studies by Chantrenne (38) and by Petermann and co-workers (39) on similar material have demonstrated that still smaller, essentially macromolecular, components of the cytoplasm are especially rich in ribonucleic acid. These, it is reasonable to suppose, might represent the dense particulates of the electron microscope image described above.

FIG. 9. Thin section through marginal portion of mucous cell from rat parotid. End of nucleus is evident at *n*, mitochondria at *m* and cell membrane at *cm*. The long slender profiles represent transverse sections through flattened veiscles which tend toward parallel orientation in these cells. The vesicles or cisternae at the upper left are extensive, at least along one axis caught in the plane of section. Those in the lower left are less so and show interruptions (arrows) where the plane of section coincides with holes or fenestrae in the otherwise intact and sheet-like structure. As a result of such fenestration a cisterna becomes reticular and in the extreme may break up into parallel arrays of separate vesicles. Small dense particulates now known to be rich in RNA are especially abundant in the cytoplasmic matrix side of the membranes (× 20,000).

The problem of relating these two sets of findings and resolving their differences is one requiring simultaneous chemical and electron microscope investigation of the products of centrifugal fractionation. This Palade and Siekevitz have recently attempted (40), and while their studies are still in a preliminary phase it is important to describe the results because they clearly relate the intact structure to the isolated cell component. In brief, they have shown that the fraction of rat liver, which is generally referred to as microsomal, consists of vesicular elements identical with the vesicles of the endoplasmic reticulum. The presence of dense particulates on their surfaces further identifies them and demonstrates as well that the fraction consists of at least two components. These investigators further report that, with the aid of desoxycholate, the vesicular elements of the fraction could be destroyed and that the granules or dense particulates can then be sedimented out and analyzed separately. The results of such analyses have demonstrated that the particles and not the vesicles contain the RNA of the microsomal fraction, and therefore confirm the conclusions drawn from the microscopy of intact cells.

SUMMARY

An attempt has been made to show that certain submicroscopic components and patterns of structure repeat from cell to cell and organism to organism at levels of resolution provided by the electron microscope. Cilia and mitochondria in their fine structure are excellent examples. Further cell components of some special interest in

FIG. 10. Micrograph showing parallel orientation of cisternae along basal margin of albuminous cell of rat parotid. The apical portion of the cell was filled with secretory granules at the time of fixation. When closely packed in this manner the cisternae are generally thinner than otherwise, and here have a thickness of approximately 350 Å. The membrane along the basal surface of the cell is at *cm* (× 27,000).

FIG. 11. Micrograph showing small dense particulates associated with surfaces of endoplasmic reticulum in pancreas cell of rat. Extended fixation with OsO_4 has accentuated the image of the particulates by removing other components of the cytoplasmic matrix. In pancreas cells and other types involved in active protein synthesis the particulates are as a rule closely applied to the membranes, as here. They are now known to be rich in RNA (40) and are probably responsible for the pronounced basophilia of the reticulum characteristic of these cells (× 71,000).

this regard are a finely-divided, vacuolar system of the cytoplasm and a frequently associated small particulate. The vacuolar system appears frequently in the form of a complex reticulum, but this configuration is far from stable and under certain conditions the system may break down into separate and distinct vesicles. In this form it can be isolated from the cell as the so-called microsomal fraction. The property of basophilia that is frequently associated with this system has been shown to reside in the small ubiquitous particles which contain the greater part of the cytoplasmic RNA.

Grateful acknowledgment is made to Dr. George Palade for figure 8 and to Dr. Michael Watson for figure 3. Figures 2 and 4 are from a study with Dr. Albert Sedar currently in press.

REFERENCES

1. Fawcett, D. W. and K. R. Porter. *Anat. Rec.* 113: 539, 1952.
2. Jakus, M. A. and C. E. Hall. *Biol. Bull.* 91: 141, 1946.
3. Sedar, A. and K. R. Porter. *J. Biophys. & Biochem. Cytology.* In press.
4. Manton, I. *Symposium, Society for Exper. Biol.* 6: 306, 1952.
5. Randall, J. T. and M. H. G. Friedlaender. *Exper. Cell Research* 1: 1, 1950.
6. Hodge, A. J. *Austr. J. Scient. Research* Ser. B. 2: 368, 1949.
7. Watson, M. L. Univ. of Rochester Atomic Energy Project, *UR-185*, 1952. (Unclassified.)
8. Challice, C. E. *Proc. Soc. for Study of Fertility* (Gt. Brit.) 4: 21, 1952.
9. Palade, G. E. *Anat. Rec.* 114: 427, 1952.
10. Palade, G. E. *J. Histochem. & Cytochem.* 1: 188, 1953.
11. Sjöstrand, F. S. *Nature* 171: 30, 1953.
12. Steinmann, E. *Exper. Cell Research* 3: 367, 1952.
13. Wolken, J. J. and G. E. Palade. *Ann. New York Acad. Sci.* 56: 873, 1953.
14. Sager, R. and G. E. Palade. *Exper. Cell Research* 7: 584, 1954.
15. Sjöstrand, F. S. *J. Cell. & Comp. Physiol.* 42: 15, 1953.
16. Bennett, H. S. and K. R. Porter. *Am. J. Anat.* 93: 61, 1953.
17. Chapman, G. B. *J. Morphol.* 95: 237, 1954.
18. Huxley, H. E. *Biochim. et Biophys. Acta* 12: 387, 1953.
19. Hodge, A. J. *J. Biophys. & Biochem. Cytology.* In press.
20. Porter, K. R. *J. Roy. Micros. Soc.* In press. Proceedings of International Congress on Electron Microscopy, London, 1954.
21. Porter, K. R. *Henry Bunting Memorial Volume on Analytical Cytology*, edited by S. L. Paley. New Haven: Yale, 1955.
22. Palade, G. E. *J. Exper. Med.* 95: 285, 1952.
23. Porter, K. R. and J. Blum. *Anat. Rec.* 117: 685, 1953.
24. Sjöstrand, F. S. *Experientia* 9: 114, 1953.
25. Claude, A. *J. Exper. Med.* 84: 51, 1946.
26. Porter, K. R., A. Claude and E. F. Fullam. *J. Exper. Med.* 81: 233, 1945.
27. Porter, K. R. *J. Exper. Med.* 97: 727, 1953.
28. Palade, G. E. and K. R. Porter. *J. Exper. Med.* 100: 641, 1954.
29. Dalton, A. J. *Am. J. Anat.* 89: 109, 1951.
30. Bernhard, W., F. Hagenau, A. Gautier and C. Oberling. *Z. Zellforsch.* 37: 281, 1952.
31. Weiss, J. M. *J. Exper. Med.* 98: 607, 1953.
32. Porter, K. R. *J. Histochem. & Cytochem.* 2: 346, 1954.
33. Bernhard, W., A. Gautier and C. Rouiller. *Arch. Anat. micr. et morph. exper.* 43: 236, 1954.
34. Garnier, C. *J. anat. et physiol.* (Paris) 36: 22, 1900.
35. Palay, S. L. and G. E. Palade. *J. Biophys. & Biochem. Cytology* 1: 69, 1955.
36. Palade, G. E. *J. Biophys. & Biochem. Cytology* 1: 59, 1955.
37. Hogeboom, G. H., W. C. Schneider and G. Palade. *J. Biol. Chem.* 172: 619, 1948.
38. Chantrenne, H. *Biochim. et Biophys. Acta* 1: 437, 1947.
39. Petermann, M. L., N. A. Mizen and M. G. Hamilton. *Cancer Research* 13: 372, 1953.
40. Palade, G. E. and P. Siekevitz. *Federation Proc.* 14: 262, 1955.

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Amino Acid Metabolism[1]

Chairman: D. D. VAN SLYKE

TRANSAMINATION IN AMINO ACID METABOLISM

ALTON MEISTER

From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

TRANSAMINATION is a chemical reaction in which an amino group is transferred from one molecule to another without the intermediate participation of ammonia. Herbst and Engel, in 1934, were the first to demonstrate such reactions using boiling aqueous solutions of amino and keto acids (1). Several years later, as a result of the pioneer studies of Braunstein and Kritzmann (2) and of Cohen (3), the occurrence of enzymatic transamination was recognized. Until recently, transamination was considered to be limited to reversible reactions between glutamate and *a*) oxalacetate and *b*) pyruvate. It may be noted, however, that experimental evidence consistent with a more significant role of transamination reactions was available from several approaches.

Evidence for amination of α-keto acids to the analogous amino acids was obtained many years ago in perfusion experiments (4) and also in whole animal studies in which an administered α-keto acid led to the urinary excretion of the corresponding N-acetyl amino acid (5). The ability of α-keto acids to serve in place of the analogous amino acids in metabolic reactions and in supporting growth has been shown in a great number of experiments. One of the earliest was that of Canzanelli, Guild, and Harington (6), who observed that the α-keto analogue of thyroxine exhibited thyroxine activity. More recent work has revealed rapid incorporation of administered ammonium and amino group nitrogen into almost all of the amino acids (7), and the similarly rapid biological labilization of the α-hydrogen atom of amino acids (8).

Although these investigations were (and still are) consistent with the widespread occurrence and significance of transamination reactions, it was generally believed until recently that the transamination reaction was of relatively limited scope and metabolic significance (7, 9, 10). The general importance of transamination has become evident from *a*) studies on the degradation and synthesis of individual amino acids using isotopic and mutant techniques, and *b*) the demonstration of a great variety of enzymatic transamination reactions. The development of improved techniques of amino acid identification and quantitation has had a great influence on work in this area.

The occurrence of an α-ketoglutarate-amino acid reaction has now been shown in a variety of animal and plant tissues and in a number of microorganisms. Virtually every known natural amino acid (and several which are as yet known only as products of synthetic organic chemistry) has been found to participate in enzymatic transamination. The task of those interested in such reactions is at least two fold: *a*) to elucidate the nature of these reactions and the associated catalytic components, and *b*) to evaluate, if possible, the significance of these reactions in metabolism.

Some of the transamination reactions which have been observed are listed in table 1. In addition to reversible reactions between α-ketoglutarate and the common natural L-α-amino acids, a number of reactions not involving dicarboxylic amino or keto acids has been reported. Transamination of ω-amino acids (and aldehydes), amino acid amides, and D-amino acids has also been observed. A reaction between

[1] San Francisco, April 12, 1955.

TABLE 1. SOME REPRESENTATIVE TRANSAMINATION REACTIONS*

(*1*) α-Ketoglutarate + L-α-amino acid ⇌ L-glutamate + α-Keto Acid
(*2*) α-Ketoglutarate + γ-aminobutyric acid ⇌ L-glutamate + succinic semialdehyde
(3) α-Ketoglutarate + D-α-amino acid ⇌ D-glutamate + α-Keto acid
(*4*) L-Glutamine + α-keto acid → α-Ketoglutaramate + L-α-amino acid
(*5*) L-Asparagine + α-Keto acid ⇌ α-Ketosuccinamate + L-α-amino acid
(*6*) L-Ornithine + α-Keto acid ⇌ L-α-amino acid + L-glutamic-γ-semialdehyde
(7) L-Valine + pyruvate ⇌ L-alanine + α-Ketoisovalerate
(*8*) L-Serine + pyruvate ⇌ L-alanine + β-hydroxypyruvate
(*9*) N-Acetyl-L-glutamic-γ-semialdehyde + L-glutamate → α-Ketoglutarate + α-N-acetyl-L-ornithine
(*10*) Imidazole acetol phosphate + L-glutamate ⇌ L-Histidinol phosphate + α-Ketoglutarate

* See also (11).

α-ketoglutarate and imidazole acetol phosphate yielding glutamate and histidinol phosphate represents a new type of reaction involving phosphate esters. Time does not permit a detailed consideration of each of these reactions. This presentation will therefore be limited to a discussion of several representative enzymatic transamination reactions, which have been studied in our laboratory.

TRANSAMINASES OF ESCHERICHIA COLI

A great amount of the work which has been done on the biosynthesis of amino acids has been carried out with *E. coli* and mutants of this organism. That preparations of *E. coli* were capable of catalyzing a variety of α-ketoglutarate-amino acid transamination reactions was first demonstrated by Feldman and Gunsalus (12). In an attempt to gain information concerning the nature of the transaminases of such cells, Dr. D. Rudman and I fractionated cell-free extracts of *E. coli* by means of ammonium sulfate precipitation and selective adsorption and elution from calcium phosphate gel (13). By these means two major transaminase fractions were obtained. One of these (fraction B) catalyzed reversible transamination reactions between α-ketoglutarate and isoleucine, leucine, valine, and to a lesser extent methionine, phenylalanine, and tyrosine. This fraction exhibited no activity toward tryptophan or aspartate. The other fraction (A) catalyzed reactions between α-ketoglutarate and aspartate, tryptophan, tyrosine, phenylalanine, and to a lesser extent methionine and leucine, but *did not catalyze reactions involving valine or isoleucine*, two of the most susceptible substrates of fraction B. In addition to α-ketoglutarate-amino acid reactions, each fraction catalyzed transamination between its susceptible amino acids and their α-keto analogues. For example, fraction B catalyzed reactions between leucine and α-keto-β-methylvalerate and between isoleucine and α-ketoisovalerate. It is possible to account for such reactions in terms of coupled α-ketoglutarate-amino acid reactions, which might take place in the presence of small quantities of glutamate or α-ketoglutarate. However, there is evidence in support of the concept that such amino group transfers take place directly. The reactions given in table 2 have been studied in extensively dialyzed cell-free extracts of *E. coli* and also with mixtures of fractions A and B. *Reaction 5* may take place directly or (provided that α-ketoglutarate or glutamate are present), may represent the sum of *reactions 4* and *2*. The failure of *reaction 3* to take place suggests that α-ketoglutarate and glutamate are not present in sufficient concentrations to permit the occurrence of such coupled reactions. These observations are consistent with the concept that a single transaminase can catalyze transamination between several amino acid-keto acid pairs.

Unequivocal evidence for the occurrence of direct transamination between monocarboxylic amino and keto acids arose from the isolation of a separate enzyme (fraction C), which catalyzed reversible transamination between valine and pyruvate (or α-ketobutyrate). This fraction did not catalyze transamination between glutamate (or aspartate) and α-ketoisovalerate or pyruvate, thus excluding the possibility of a coupled reaction. Cell-free extracts of an *E. coli* mutant which required L-isoleucine for growth but which did not respond to *d*-α-keto-β-methylvalerate, exhibited a specificity which was almost identical with that of fraction A obtained from the wild strain of origin; i.e., activity corresponding to fraction B was lacking. On the basis of the one gene-one-enzyme hypothesis, it may be concluded that fraction B is a single enzyme. Fraction A may possibly represent more than one enzyme; further purification will be necessary to clarify this point.

Certain other aspects of this problem are also

of interest. As indicated above, the isoleucineless mutant which did not utilize the α-keto analogue of isoleucine for growth, did not possess an enzyme activity capable of synthesizing isoleucine from its keto analogue. Since this enzyme is apparently identical with one which catalyzes valine formation from α-ketoisovalerate, an absolute growth requirement for valine might also be expected. Actually this mutant exhibits a partial requirement for valine, but is capable of synthesizing some valine, probably because it possesses enzyme activity corresponding to fraction C, i.e., the valine-alanine, α-aminobutyric acid transaminase. When this isoleucineless mutant is grown on valine-deficient media, the activity of the valine-alanine, α-aminobutyric acid system increases, and the organism no longer requires valine (14). However, isoleucineless mutants which require supplementation with valine for optimal growth respond equally well to equivalent concentrations of either alanine or α-aminobutyric acid (13).

There are, therefore, two transamination systems for valine synthesis in *E. coli*, and it may be predicted that a genetic block of valine synthesis at the stage of α-ketoisovalerate would require two mutations. It is interesting to note that at least three other amino acids are synthesized in *E. coli* via the corresponding α-keto acid, *i.e.*, phenylalanine, tyrosine, and leucine. Transamination of these amino acids is catalyzed by both fractions A and B, although, as noted above, reactions of the aromatic amino acids proceed considerably more rapidly with fraction A. However, of the several amino acids which are synthesized by transamination from the corresponding α-keto acids, isoleucine is the only one for which there is a single transaminase. This finding is consistent with the fact that thus far the only mutant known to be blocked at the stage of transamination is an isoleucineless mutant.

AMINO ACID AMIDE TRANSAMINASES

The presence of relatively high concentrations of free glutamine in the tissues and body fluids of mammals suggests a significant metabolic role. Indeed, a number of studies have indicated that glutamine functions in formation of urinary ammonia, synthesis of purines, formation of amino sugars, metabolism of nerve tissue, detoxification, and other reactions. Asparagine is also found in mammalian tissues, although the concentration of this amide is much lower than that of glutamine, and less is known of its metabolic function.

The ability of glutamine to participate in transamination reactions with α-keto acids was first observed in 1950 (15). Preparations of rat liver and kidney catalyze the reaction, which leads to the formation of a new amino acid, α-ketoglutarate, and ammonia. The intermediate formation of α-ketoglutaramate, the α-keto analogue of glutamine, was suspected inasmuch as *a*) substitution of glutamine by glutamate resulted in lowered transamination rates, suggesting that deamidation did not precede transamination, and *b*) α-ketoglutaramate prepared by an independent procedure was promptly deamidated to α-ketoglutarate by the enzyme preparation (16). An analogous reaction catalyzed by an apparently different hepatic enzyme was observed between asparagine and α-keto acids (17); the α-keto analogue of asparagine, α-ketosuccinamate, was also susceptible to hydrolysis by the enzyme. Further evidence in support of the two-step mechanism proposed for these reactions was obtained in studies with the glutamine transaminase and γ-methylglutamine (18). The latter compound (as well as the naturally occurring γ-methyleneglutamine) transaminated readily with α-keto acids, but no ammonia was formed in the reaction. α-Keto-γ-methylglutaramic acid was shown to be a product of the γ-methylglutamine-α-keto acid reaction, and a preparation of α-keto-γ-methylglutaramic acid prepared by another procedure was not deamidated by the enzyme. These reactions may therefore be represented as follows.

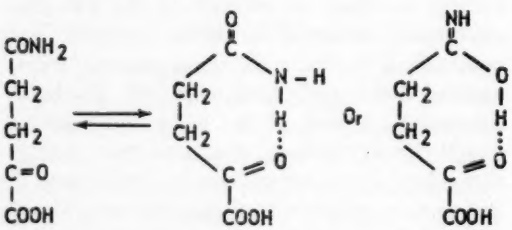
(*1*) Glutamine + α-Keto acid → α-Amino acid + α-ketoglutaramate

(*2*) α-Ketoglutaramate + H_2O → α-Ketoglutarate + NH_3

(*3*) γ-Methylglutamine + α-keto acid → α-amino acid + α-keto-γ-methylglutaramate

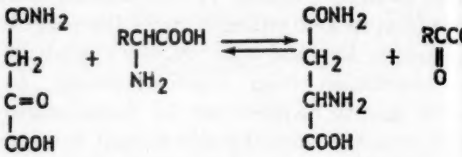
The α-keto analogues of glutamine, asparagine, and γ-methylglutamine were prepared by a procedure used previously in this laboratory for the preparation of a large number of other α-keto acids, namely by enzymatic oxidative deamination of the corresponding amino acids followed by isolation in the pure state of the keto acid or its salt (19). It was found that the preparations of α-ketoglutaramate and α-ketosuccinamate obtained by this procedure differed from typical α-keto acids in that *a*) they formed 2,4-dinitro-

phenylhydrazones very slowly, and *b*) they were not decarboxylated by hydrogen peroxide at pH 5. However, it was possible by treatment with alkali to convert these relatively unreactive forms to products which gave hydrazones rapidly and which underwent peroxide decarboxylation. It was also found that only the reactive forms were susceptible to enzymatic deamidation (16). Further study of this class of compounds has led to the preparation of a series of similar products, e.g., α-ketoadipamic acid, α-keto-N-dimethylglutaramic acid, γ-(α-ketoglutaryl)-glycine, β-oxalacetylglycine, and it has become evident that the tendency to exist in such unreactive forms is restricted to α-keto acid-ω-amides which possess carbon chains of 4 or 5 atoms and at least one unsubstituted amide hydrogen atom (18, 20). Recent studies in our laboratory by Dr. T. T. Otani suggest that α-ketoglutaramic acid exists as a cyclic structure formed by intramolecular hydrogen bonding (20):



Similar structures may be written for α-ketosuccinamic acid and other compounds of this series. This formulation rests on evidence which appears to exclude other structures (e.g., enolic forms, imides, lactams) and on infrared absorption data which support the proposed configuration. γ-(α-ketoglutaryl)-glycine and γ-(α-ketoglutaryl)-L-alanine exist predominantly in the cyclic state, while stable preparations of β-oxalacetylglycine and β-oxalacetyl-L-alanine in the open chain form may be readily obtained. Similarly, while α-ketoglutaramic acid exists mainly in the cyclic form even in alkaline (pH 9) solution, neutral solutions of α-ketosuccinamate in the reactive form can be prepared (16).

We have not as yet been able to demonstrate the formation of glutamine from α-ketoglutaramate by enzymatic transamination (although this reaction has been carried out nonenzymatically with pyridoxamine at 100°), possibly because of the tendency of α-ketoglutaramate to exist in cyclic form, or because of its susceptibility to attack by the amidase. On the other hand, the asparagine-α-keto acid transamination reaction appears to be reversible, i.e., α-ketosuccinamic acid readily transaminates with a variety of L-α-amino acids to yield L-asparagine (21):



The demonstration of this reaction provides further support for the two-step mechanism proposed for the asparagine-α-keto acid reaction, and raises the question as to whether this reaction may be involved in the mechanism of asparagine synthesis. The formation of α-ketosuccinamate has thus far not been demonstrated (except from asparagine itself by transamination or oxidation). An amidase present in liver and other tissues, which catalyzes the deamidation of α-ketosuccinamate, α-ketoglutaramate, glutaramate, and succinamate, also catalyzes transfer reactions involving hydroxylamine and the last three of these amides (22). The possible role of this system in α-ketosuccinamate formation remains to be investigated.

The glutamine-α-keto acid system is characterized by a very wide specificity for the α-keto acid; on the other hand, only a few amino group donors are active. Thus, L-glutamine may not be replaced by its enantiomorph or by its higher (homoglutamine) or lower (α-aminomalonamic acid, asparagine) homologues. Although γ-methyl and γ-methylene substituted glutamines are active, the α-methyl and β-methyl derivatives of glutamine are inert. γ-Glutamylmethylamide was the only active amide-substituted derivative; in this case methylamine was formed in place of ammonia. While certain glutamine derivatives were inactive as amino donors (e.g., homoglutamine, γ-glutamyldimethylamide), their α-keto analogues were aminated in this system in the presence of glutamine (18). Recently, Dr. T. T. Otani in our laboratory observed that β-oxalacetylglycine and β-oxalacetyl-L-alanine were capable of transamination with glutamine to yield β-aspartylglycine and β-aspartylalanine (20). The demonstration of these reactions serves to widen the known scope of enzymatic transamination to include compounds which possess a peptide bond. However, transamination of α-peptides has not been unequivocally demonstrated. A study of the α-keto analogues of γ-glutamyl- and β-aspartyl-peptides is in progress.

TABLE 2. SEVERAL TRANSAMINATION REACTIONS CATALYZED BY E. COLI PREPARATIONS

No.	Reaction	Transamination Rate*
(1)	Tyrosine + α-ketoglutarate $\rightleftharpoons$ p-hydroxyphenylpyruvate + glutamate	10.3
(2)	Glutamate + α-keto-β-methylvalerate $\rightleftharpoons$ isoleucine + α-ketoglutarate	8.1
(3)	Tyrosine + α-keto-β-methylvalerate $\rightleftharpoons$ isoleucine + p-hydroxyphenylpyruvate	0
(4)	Leucine + α-ketoglutarate $\rightleftharpoons$ glutamate + α-ketoisocaproate	16.9
(5)	Leucine + α-keto-β-methylvalerate $\rightleftharpoons$ α-ketoisocaproate + isoleucine	14.4

* μM/mg of enzyme preparation N/hr. (13).

The fact that more than 30 α-keto acids are capable of transamination with glutamine and asparagine suggests that these systems may be of general significance in amino acid metabolism in liver and kidney, and it is probable that transamination represents at least one of the reactions which compete for glutamine and asparagine. The presence of an appreciable concentration of free glutamine in liver (23) suggests that the glutamine-α-keto acid system may function so as to remove free α-keto acids. The glutamine transaminase system, in contrast to the glutamate-amino acid systems, would appear to be irreversible because of rapid deamidation of α-ketoglutaramate. These considerations suggest that the glutamine transaminase may be primarily involved in amino acid synthesis rather than in amino acid breakdown.[2]

Both pyridoxal phosphate and pyridoxamine phosphate are now known to be the coenzymes for transaminase; a recent study has dealt with the problems of coenzyme activation, binding, and inhibition (24). Many of the transaminases which have been described have been shown to require a phosphorylated vitamin B_6 derivative. In such studies, transaminases have been resolved by fractionation procedures or by isolation from vitamin B_6 deficient tissues or cells. Previous attempts to demonstrate a coenzyme requirement for the glutamine transaminase by fractionation or by utilizing the livers of vitamin B_6 deficient rats were not successful (25). It was found that while the cysteine desulfhydrase and glutamate-alanine transaminase activities of the livers of vitamin B_6 deficient rats were markedly reduced, there was no significant lowering of glutamine transaminase activity. This result suggested that either vitamin B_6 was not a coenzyme for the glutamine transaminase, or that the vitamin B_6-coenzyme was very tightly bound by this enzyme.

Recently it was reported that patients treated with isonicotinic acid hydrazide excreted large quantities of bound vitamin B_6, and that such patients experienced a neuritis amenable to therapy with vitamin B_6 (26). Although clinical application generally follows laboratory study, in this instance the clinical observations suggested a laboratory approach. We therefore gave large doses of isonicotinic acid hydrazide to several groups of rats. Such treatment reduced the glutamine transaminase activity of the liver, and purified enzyme preparations obtained from these livers exhibited a considerable increase in activity after addition of either pyridoxal phosphate or pyridoxamine phosphate (27). Isonicotinic acid hydrazide has been reported to inhibit pyridoxal phosphate enzyme systems *in vitro* (28, 29). The *in vivo* activity of this compound may probably be ascribed to its ability to combine with pyridoxal phosphate to form the corresponding hydrazone. The present experiments suggest that the glutamine transaminase, like other transaminases, is a vitamin B_6 enzyme, and also that under appropriate conditions, the deamidation of glutamine may be made dependent on the presence of vitamin B_6 (27).

SIGNIFICANCE OF TRANSAMINATION IN METABOLISM OF OTHER AMINO ACIDS

In this discussion I have attempted to review in some detail studies on the role of transamination in the biosynthesis of amino acids in a bacterial organism, and experiments on amino acid amide transamination in animal tissues (see

[2] Some experiments recently carried out at the Clinical Center of the National Institutes of Health in collaboration with Dr. Sidney Udenfriend (National Heart Institute) and Dr. Samuel Bessman (Department of Pediatrics, University of Maryland Medical School) yielded results which may be of interest in connection with transamination. Patients with phenylpyruvic oligophrenia were given relatively large doses of glutamine, glutamate, or asparagine. After glutamine administration, the urinary excretion of phenylpyruvate fell to about ⅓ of the control value. Glutamate and asparagine also reduced the excretion of phenylpyruvate, albeit to a smaller extent. Although we cannot yet be certain as to the interpretation of these effects, the possibility that the lowered phenylpyruvate excretion is due to transamination between this keto acid and the administered amino acid is attractive.

TABLE 3. TRANSAMINATION IN AMINO ACID METABOLISM (REFERENCES TO LITERATURE)*

Amino Acid	Synthesis	Degradation
Glutamate	(9, 10)	(9, 10)
Aspartate	(9, 10)	(9, 10)
Alanine	(9, 10)	(9, 10)
Isoleucine	(13, 14, 34, 35)	(38)
Valine	(13, 14, 34, 35)	(36, 37)
Leucine	(13)	(39)
Glycine	(40)	
Tyrosine	(41)	(42, 43, 44)
Lysine		(45)
Ornithine	(46)	(47)
Tryptophan		(48)
Phenylalanine	(13, 41)	
Glutamine		(49)
Asparagine		(49)
Diiodotyrosine		(50)
Histidine	(51)	
Methionine		
Cysteine		(52)
β-Alanine		(53, 54)
γ-Aminobutyric acid		(53, 54)
Serine	(55)	(55)

* See also (11).

table 3). Although a number of other transamination systems are known, it is clear that the available data constitute only a small sample of the information necessary for a complete understanding of the metabolic significance of transamination. The first transamination reactions to be described, the glutamate-aspartate and glutamate-alanine reactions, provide important links between these amino acids and the corresponding keto acid intermediates of the citric acid cycle. Recent work has demonstrated the occurrence of many other glutamate-amino acid transamination reactions, and the deamination of L-amino acids observed in early work (5, 30, 31) may plausibly be explained in terms of coupled transamination-glutamic dehydrogenase reactions:

$$\alpha\text{-Ketoglutarate} + \text{L-Amino acid} \rightleftharpoons \text{L-Glutamate} + \alpha\text{-Keto acid}$$

$$\text{L-Glutamate} + \tfrac{1}{2}\,O_2 \rightleftharpoons \alpha\text{-Ketoglutarate} + NH_3$$

At this time there appears to be little evidence for the existence of a mammalian L-amino acid oxidase of appreciable activity.

Recent studies suggest that the degradation of leucine, isoleucine, valine, and tyrosine proceeds via the corresponding α-keto acids, and that this conversion takes place by transamination. As discussed above, transamination appears to be the final step in the biosynthesis of these amino acids. Transamination reactions are also involved in the interconversions between glutamate, ornithine, and proline, and in the synthesis of histidine and glycine. The metabolism of cysteine may involve transamination of cysteine sulfinic acid, cysteic acid, or of cysteine itself. The degradation of kynurenine and of lysine may also require transamination.

It is thus apparent that the degradation of many amino acids requires a preliminary separation of the α-amino group from the amino acid carbon skeleton by transamination, and that the synthesis of amino acids frequently involves a reversal of this reaction. At this time it appears that the 'major' metabolic pathways of several amino acids (e. g., tryptophan, methionine, phenylalanine) do not involve transamination, although these amino acids are known to participate in such reactions *in vitro*. It may be observed that present designations of 'major' pathways of metabolism may not be final, and that pathways of minor significance from a quantitative standpoint may be of great physiological importance. Thus, a relatively small fraction of α-ketoisovalerate is diverted to pantoic acid formation (32). The presence of phenylacetylglutamine in normal human urine (33) suggests that normally some phenylalanine may transaminate to phenylpyruvate, a pathway which is exaggerated in phenylpyruvic oligophrenia. The rapid incorporation of ammonia into almost all of the amino acids of animal and plant tissues is consistent with the widespread occurrence of transamination reactions. The question as to whether such amino acids as methionine, histidine, and tryptophan may be metabolized to some extent by pathways involving transamination to the analogous α-keto acids remains to be investigated.

REFERENCES

1. HERBST, R. M. AND L. L. ENGEL. *J. Biol. Chem.* 107: 505, 1934.
2. BRAUNSTEIN, A. E. AND M. G. KRITZMANN. *Enzymologia* 2: 129, 1937.
3. COHEN, P. P. *Biochem. J.* 33: 1478, 1939.
4. EMBDEN, G. AND E. SCHMITZ. *Biochem. Ztsch.* 29: 423, 1910; 38: 393, 1912.
5. KNOOP, F. *Ztschr Physiol. Chem.* 67: 489, 191
6. CANZANELLI, A., R. GUILD AND C. R. HARINGTON. *Biochem. J.* 29: 1617, 1935.

7. SCHOENHEIMER, R. *The Dynamic State of Body Constituents*. Cambridge; Harvard, 1949.
8. SPRINSON, D. B. AND D. RITTENBERG. *J. Biol. Chem.* 184: 405, 1950.
9. BRAUNSTEIN, A. E. In *Advances in Protein Chemistry*. Vol. III. New York: Academic Press, 1947, p. 1.
10. COHEN, P. P. In: *The Enzymes*, Vol. I, edited by Sumner-Myrback. New York: Academic Press, 1951, p. 1040.
11. MEISTER, A. *Advances Enzymol.* 16: 185, 1955.
12. FELDMAN, L. I. AND I. C. GUNSALUS. *J. Biol. Chem.* 187; 821, 1950.
13. RUDMAN, D. AND A. MEISTER. *J. Biol. Chem.* 200; 591, 1953.
14. ADELBERG, E. A. AND H. E. UMBARGER. *J. Biol. Chem.* 205: 475, 1953.
15. MEISTER, A. AND S. V. TICE. *J. Biol. Chem.* 187: 173, 1950.
16. MEISTER, A. *J. Biol. Chem.* 200: 571, 1953.
17. MEISTER, A., H. A. SOBER, S. V. TICE AND P. E. FRASER. *J. Biol. Chem.* 197: 319, 1952.
18. MEISTER, A. *J. Biol. Chem.* 210: 17, 1954.
19. MEISTER, A. *J. Biol. Chem.* 197: 309, 1952.
20. OTANI, T. T. AND A. MEISTER. Abstr., 127th Meeting, American Chemical Society, Cincinnati, Ohio, 1955.
21. MEISTER, A. AND P. E. FRASER. *J. Biol. Chem.* 210: 37, 1954.
22. MEISTER, A., L. LEVINTOW, R. E. GREENFIELD AND P. A. ABENDSCHEIN. Abstr. 127th Meeting, American Chemical Society, Cincinnati, Ohio, 1955; *J. Biol. Chem.* In press.
23. WAELSCH, H. *Advances Enzymol.* 13: 237, 1952.
24. MEISTER, A., H. A. SOBER AND E. A. PETERSON. *J. Biol. Chem.* 206: 89, 1954.
25. MEISTER, A., H. P. MORRIS AND S. V. TICE. *Proc. Soc. Exper. Biol. & Med.* 82: 301, 1953.
26. BIEHL, J. P. AND R. W. VILTER. *Proc. Soc. Exper. Biol. & Med.* 85: 389, 1954.
27. MEISTER, A. AND P. E. FRASER. Unpublished data.
28. YONEDA, M. AND N. ASANO. *Science* 117: 277, 1953.
29. YONEDA, M., N. KATO AND M. OKAJUNA. *Nature* 170: 803, 1952.
30. NEUBAUER, O. *Deut. Arch. klin. Med.* 95: 211, 1909.
31. KREBS, H. A. *Biochem. J.* 29: 1620, 1935.
32. MAAS, W. K. AND H. K. VOGEL. *J. Bact.* 65: 388, 1953.
33. STEIN, W. H., A. C. PALADINI, C. H. W. HIRS AND S. MOORE. *J. Am. Chem. Soc.* 76: 2848, 1954.
34. BONNER, D., E. L. TATUM AND G. W. BEADLE. *Arch. Biochem.* 3: 71, 1943.
35. UMBARGER, H. E. AND E. A. ADELBERG. *J. Biol. Chem.* 192: 883, 1951.
36. FONES, W. S., T. P. WAALKES AND J. WHITE. *Arch. Biochem. Biophys.*, 32: 89, 1951.
37. PETERSON, E. A., W. S. FONES AND J. WHITE. *Arch. Biochem. Biophys.* 36: 323, 1952.
38. COON, M. J., N. S. B. ABRAHAMSEN AND G. S. GREENE. *J. Biol. Chem.* 199: 75, 1952.
39. COON, M. J. AND S. GURIN. *J. Biol. Chem.* 180: 1159, 1949.
40. WEINHOUSE, S. AND B. FRIENMANN. *J. Biol. Chem.* 191: 707, 1951.
41. DAVIS, B. D. *Advances Enzymol.* 16: 247, 1955.
42. SCHEPARTZ, B. *J. Biol. Chem.* 193: 293, 1951.
43. KNOX, W. E. AND M. LEMAY-KNOX. *Biochem. J.* 49: 686, 1951.
44. LA DU, B. N., JR. AND D. M. GREENBERG. *J. Biol. Chem.* 190: 245, 1951.
45. ROTHSTEIN, M. AND L. I. MILLER. *J. Am. Chem. Soc.* 75: 4347, 1953; 76: 1459, 1954.
46. VOGEL, H. J. *Amino Acid Metabolism*. Baltimore: Johns Hopkins Press, 1955, p. 335.
47. STETTEN, M. R. *Amino Acid Metabolism*. Baltimore: Johns Hopkins Press, 1955, p. 277.
48. MILLER, I. L., M. TSUCHIDA AND E. A. ADELBERG. *J. Biol. Chem.* 203: 205, 1953.
49. MEISTER, A. *Science* 120: 43, 1954.
50. TONG, W., A. TAUROG AND L. L. CHAIKOFF. *J. Biol. Chem.* 207: 59, 1954.
51. AMES, B. *Amino Acid Metabolism*. Baltimore: Johns Hopkins Press, 1955, p. 357.
52. SINGER, T. P. *Amino Acid Metabolism*. Baltimore: Johns Hopkins Press, 1955, p. 558.
53. ROBERTS, E. AND A. M. BREGOFF. *J. Biol. Chem.* 201: 393, 1953.
54. BESSMAN, S. P., J. ROSSEN AND E. C. LAYNE. *J. Biol. Chem.* 201: 385, 1953.
55. SALLACH, H. J. *Amino Acid Metabolism*. Baltimore: Johns Hopkins Press, 1955, p. 782.

DISCUSSION

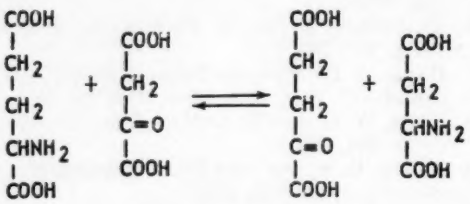
PHILIP P. COHEN

From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin

THIS brief discussion will be limited to a few general considerations of Dr. Meister's review of transamination and in the main will emphasize the areas in need of further study. Some of these remarks will simply emphasize what Dr. Meister has already briefly referred to and others will supplement some aspects which time, I am certain, has prevented Dr. Meister from including in his presentation.

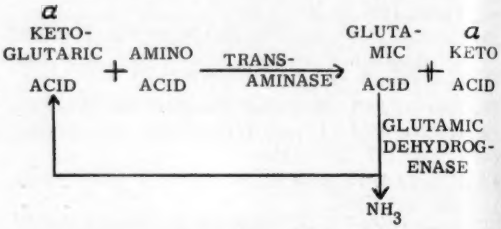
It is clear from Dr. Meister's review that a large number of different transaminases exist and that the majority of these enzymes are in need of purification so as to permit better definition of substrate specificity, kinetics, etc.

One problem which must be mentioned but which in all probability will not be readily resolved at this time, is that of nomenclature. While the term transaminase is generally accepted as suitable for the type of enzyme, the more detailed reference to a particular transaminase has involved the use of a variety of names. In a review by the author (1) it was suggested that, in keeping with the tradition that the name of an enzyme should reveal as far as possible the nature of the substrate or substrates acted upon, the specific transaminases should be named by the prefix of the substrate pair under study. Thus glutamic-oxalacetic transaminase would describe the enzyme which catalyzes the following reaction:



Dr. Meister has preferred to use the term glutamic-aspartic transaminase to describe this enzyme. This terminology has the advantage of indicating both the amino group donor and the amino acid formed. While no serious problem is posed at the moment by the two systems of nomenclature, in the interest of simplification multiple names for enzymes should be avoided. This difficulty will no doubt ultimately be resolved by the many investigators in this field on the basis of their published preferences.

The existence of a large number of different transaminases in plant, animal and microbial cells has presented the investigator with a large field to explore further. Information is particularly needed on the true rates of these reactions in different cells. Available data suggest a rather limited rate of transamination for certain amino acids. Without careful kinetic studies it is not proper to invoke transamination as an intermediate step in complex systems unless it can be established that the rate of transamination is consistent with the overall rate of the reaction under study. Adequate kinetic data are available for only a few transaminase systems. This need is particularly urgent in establishing the obligatory role of transamination in the deamination of amino acids. It has been widely accepted that most, if not all, amino acids are deaminated in the animal by the following coupled system:



While Braunstein and Azarkh (2) have reported experiments which support such a cycle, Cedrangolo (3) was not able to confirm certain of their findings. It is clear that a critical assessment of the rates of transamination of the different amino acids with α-ketoglutaric acid and the rate of glutamic acid oxidation in different tissues is necessary before this proposed cycle can be accepted as the main if not the obligatory pathway for the oxidative deamination of amino acids. It should be pointed out that in the animal, the pattern of transamination of a variety of amino acids varies considerably from organ to organ (4). It should be further emphasized that relatively little is known about the intracellular distribution of transaminases.

A most useful contribution to the study of the metabolic role of transaminases *in vitro* and *in vivo* would be the availability of specific inhibitors for the transaminase systems. To date no effective specific inhibitors are known although the search for such useful agents has not been, to the writer's knowledge, adequately explored.

REFERENCES

1. Cohen, P. P. In: *The Enzymes*, Vol. I, edited by Sumner and Myrback. New York: Academic Press, 1951, p. 1040.
2. Braunstein, A. E. *Advances in Protein Chemistry* 3: 1, 1947.
3. Cedrangolo, F. *Giorn. biochim. italo-frar.co-elvetiche, Napoli*, 21–24, April 1954. (Supplement to "La ricerca scientifica", Anno 24°—1954).
4. Cohen, P. P. Unpublished observations.

SOME ASPECTS OF AMINO ACID BIOSYNTHESIS IN MICROORGANISMS

BERNARD D. DAVIS

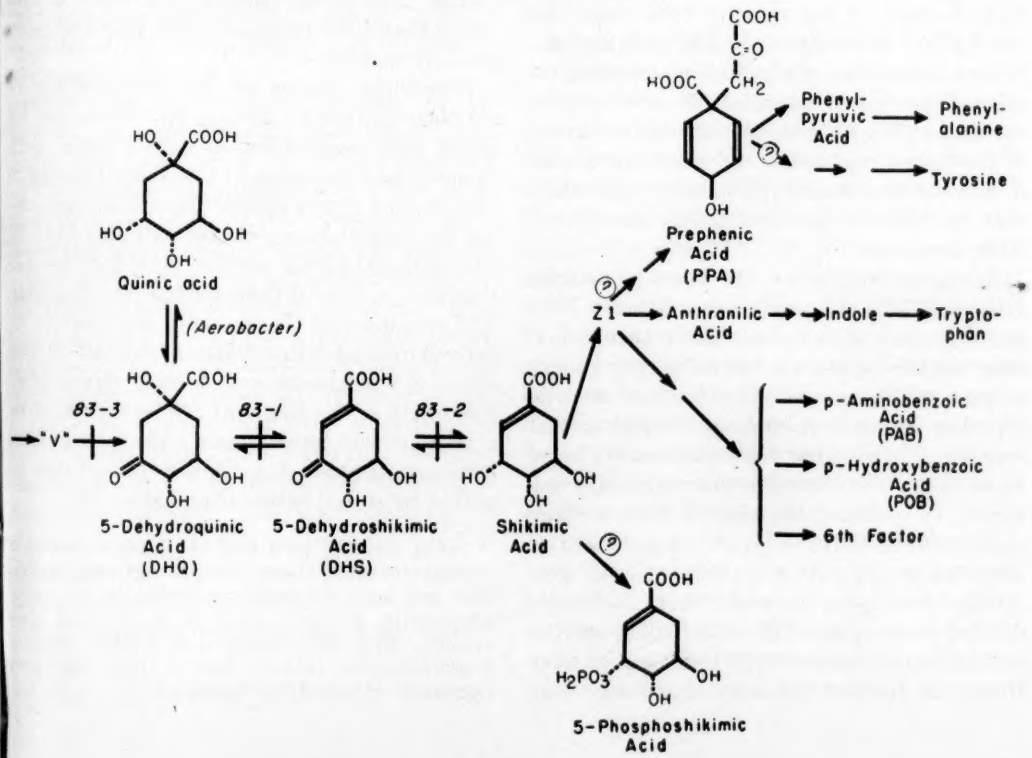
From the Department of Pharmacology, New York University College of Medicine, New York City

RECENTLY the author published a comprehensive review on intermediates in amino acid biosynthesis (1), and an exhaustive volume on amino acid metabolism has appeared (2). This paper will therefore present only brief summaries of selected portions of this material, as well as certain unpublished recent observations on aromatic biosynthesis.

The methods that have proved valuable in studying amino acid biosynthesis include the use of isotopes, antimetabolites, extracted enzymes, and auxotrophic (biosynthetically deficient) mutants of microorganisms. Of these methods, the last has been the most fruitful in revealing previously unknown intermediates. It depends on two properties of these mutants: many of them accumulate in the culture filtrate large amounts of the substrate of the blocked reaction, and many can respond nutritionally to later intermediates in the blocked pathway. It has therefore been particularly helpful to observe syntrophism (cross-feeding) between two strains with the same requirement, since one strain provides generous quantities of a previously unavailable intermediate, and the other aids in its isolation by making bioassay possible.

AROMATIC BIOSYNTHESIS

The biosynthetic studies of our group have in recent years been largely concerned with the aromatic amino acids and vitamins. This undertaking was made possible by the isolation of a wide variety of bacterial mutants requiring a mixture of tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid. With the use of these strains the following scheme has been established (3).



All of the possible intermediates shown in this scheme (except quinic acid) have been isolated from culture filtrates of mutants blocked after them. In addition, 5-dehydroshikimic acid (DHS) (4) and shikimic acid (5) are active as growth factors for mutants blocked before them. In contrast, Compound Z1 (6), 5-phosphoshikimic acid (6), and prephenic acid (7, 8) are nutritionally inactive, presumably because of permeability barriers. DHQ (5-dehydroquinic acid) (9) is also inactive, but mutants blocked before it can develop a secondary mutation that allows them to respond to it (10).

Phosphoshikimic acid may well be a side-product rather than an obligatory intermediate, since no mutants have been observed to be blocked between it and shikimic acid—i.e., all available strains that accumulate either compound also accumulate the other (6).

Prephenic acid is a compound of special interest since it is the substrate of the aromatization reaction in the biosynthesis of phenylalanine, undergoing dehydration and decarboxylation to yield phenylpyruvic acid and CO_2 (7, 8). It is extraordinarily labile to acid; even at a pH as high as 7 it demonstrably undergoes the same aromatization reaction that is normally carried out more rapidly in the cell by an enzyme.

Compound Z1 is an acid-labile conjugate of shikimic acid; it has recently been shown by Gilvarg (11) to yield pyruvic acid on hydrolysis. It thus appears to be a logical intermediate between shikimic acid and prephenic acid, containing the partly saturated ring and carboxyl group of the former compound, and also pyruvic acid in a labile attachment (presumably enol-ether) that precedes the firm attachment seen in the latter compound.

Enzymatic studies on the reaction between DHQ and DHS (12), and on that between DHS and shikimic acid (13), have shown that each of these reactions appears to be mediated by a single enzyme, which is present in extracts of the wild type but absent from mutants blocked in that reaction. Furthermore, these enzymes are found to be widely distributed in microorganisms and plants. In contrast, the enzyme that converts quinic acid to DHQ is much more limited in distribution—e.g., it is present in wild type *Aerobacter aerogenes* but absent from *Escherichia coli* and various plants (14). This finding confirms the earlier suspicion (10) that quinic acid, even though an excellent precursor of aromatic compounds in certain Aerobacter mutants, was unlikely to be a true intermediate in this path since it was neither active for nor accumulated by corresponding *E. coli* mutants.[1]

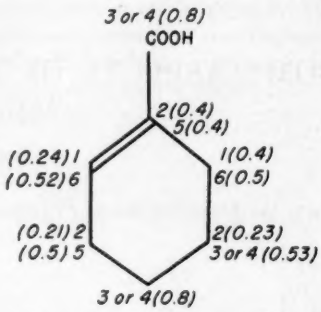


FIG. 1. Major incorporations of glucose carbon atoms into shikimate. (Whole numbers refer to carbon atoms of glucose; fractions incorporated are given in parentheses.)

NON-CYCLIC PRECURSORS

In pursuing the path behind DHQ, it was found (15) that filtrates of mutants blocked before this compound contain small amounts of an intermediate, provisionally called Compound V, which was recognized through the fact that crude extracts of appropriate mutants convert it to a readily assayed derivative, DHS. Kalan and Leitner have partly purified Compound V and shown that it is a phosphorylated keto acid, presumably open-chain.

Meanwhile, studies on the incorporation of variously C^{14}-labeled glucose into shikimate by intact cells, carried out in collaboration with Sprinson and colleagues at Columbia University (16), yielded certain general information concerning the earliest stages of this pathway (fig. 1). The carboxyl-C1-C2 portion of shikimate was shown to be derived from a glycolytic fragment. The remainder of the molecule appeared to be derived from a 4-carbon fragment in which carbon atoms 3-6 of glucose are strongly represented. The origin of this fragment can be explained by a transketolase-transaldolase series of reactions involving pentose and heptose phosphates as well as triose and hexose phosphates (16).

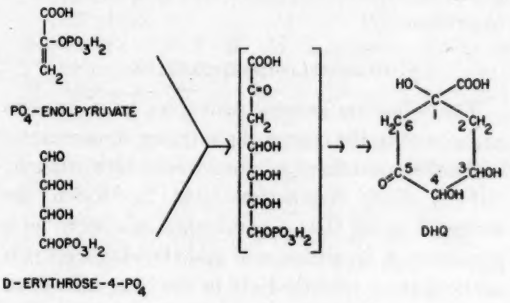
[1] It is assumed here that the same pathway is present in both these species. Actually quinic acid has been rigorously excluded as an intermediate in *E. coli* (since it is inactive with extracts that can utilize the earlier possible precursors—see below); but it has not been rigorously excluded for *Aerobacter*.

It was clear that to obtain more detailed information a cell-free system, unhampered by permeability barriers to phosphate esters, would be very useful. Since crude extracts of appropriate mutants synthesize DHS from Compound V, it seemed possible that still earlier precursors, e.g., phosphorylated carbohydrates, might also be active. This proved to be the case. Various hexose phosphates gave a yield of about 5% (17); and sedoheptulose-1,7-diphosphate (SDP)[2] yielded as much as 90% (18). Furthermore, carbon atoms 4-7 of SDP-4,5,6,7-C^{14} were converted quantitatively and exclusively into carbon atoms 3-6 of shikimic acid.

However, despite the excellent incorporation of SDP there was reason to believe that its carbon chain did not remain intact in the reactions leading to cyclization. Carbon atoms 1, 2, and 3 of SDP are derived from glucose by way of dihydroxyacetone phosphate (19), and so, in the presence of triose isomerase, would have their origin in carbon atoms (1,6)-(2,5)-(3,4) of glucose, respectively. If the intact chain of SDP underwent 2-7 cyclization the same distribution of glucose carbon atoms would be found in the carboxyl-C1-C2 portion of shikimate. But in fact the reverse order is found, with the carboxyl of shikimate arising from 3,4 of glucose rather than from 1,6 (fig. 1). It therefore appears that SDP was split into a 4-carbon and a 3-carbon fragment, the latter of which was inverted prior to recombination and cyclization.

Further enzymatic studies carried out by Sprinson and colleagues (20) have lent support to this view. Addition of fluoride to the extract (which prevents the conversion of triose phosphate to phosphoenolpyruvate) completely inhibits the synthesis of DHS from SDP. The synthesis is unimpaired, however, when phosphoenolpyruvate is added with the fluoride. Moreover, a mixture of synthetic D-erythrose-4-phosphate (kindly furnished by Dr. C. E. Ballou) and phosphoenolpyruvate can fully replace SDP in the synthesis. It therefore appears that SDP is cleaved to erythrose phosphate (C 4-7) and dihydroxyacetone phosphate (C 1-3), the latter is converted to phosphoenolpyruvate, and this compound is recondensed with the tetrose phosphate prior to cyclization. Moreover, since phosphoenolpyruvate and tetrose phosphate do not require SDP for their formation, the latter compound, despite its excellent utilization, may well not be an obligatory intermediate in aromatic biosynthesis.

The early steps in aromatic biosynthesis can now be formulated in general as follows:



OTHER AMINO ACIDS

Certain novel features revealed in several other biosynthetic pathways might be briefly mentioned.

Histidine is derived from a series of imidazole derivatives, the ring being attached to glycerol phosphate which is converted to acetol phosphate and then transaminated (21, 22). After dephosphorylation the resulting amino-alcohol, histidinol, (23) is oxidized to histidine via the amino-aldehyde, both stages of the oxidation apparently being catalyzed by the same DPN-linked enzyme (24).

Isoleucine, Valine, Leucine. In the biosynthesis of isoleucine, α-ketobutyric acid (or α-aminobutyric acid) contributes four carbon atoms and the other two are derived from active acetate. However, isotopic studies have revealed that the chain of α-ketobutyrate does not remain intact. Two somewhat different mechanisms have been proposed to account for the data: one involves an aldol and the other a ketol condensation, and both involve a pinacol-like rearrangement (25, 26). Similar observations have been made on valine synthesis, except that pyruvate takes the place of α-ketobutyrate (27). In leucine biosynthesis, isotope competition studies (28) as well as degradation of labeled leucine (29) have shown that the α-keto precursor of valine replaces its carboxyl group by an acetate group.

Ornithine, Proline. The identification of N^{α}-acetylornithine (30), accumulated by a mutant blocked before ornithine, revealed that the conversion of glutamic acid to ornithine involves a series of acetylated intermediates (31). The function of the acetyl group is apparently to prevent

[2] Generously furnished by Dr. B. L. Horecker.

spontaneous cyclization at the γ-aminoaldehyde stage in this sequence. Indeed, spontaneous cyclization of the corresponding non-acetylated derivative, glutamic γ-semialdehyde, appears to be a normal step in the conversion of glutamic acid to proline (32).

GENERAL CONSIDERATIONS

The following general principles have become prominent in the course of analyzing the aromatic and other pathways of amino acid biosynthesis.

1) A sharp distinction must be drawn between showing that a compound can serve as a precursor of an amino acid and showing that it is an obligatory intermediate in the biosynthesis of that amino acid from a general carbon source, such as glucose. In the aromatic pathway, for example, quinic acid is an excellent precursor for certain *Aerobacter* mutants, but a number of considerations suggest that it is not a normal intermediate. Similarly, various organisms can convert histidine to glutamic acid, or threonine to isoleucine, or ornithine to proline, but it is unlikely that these are normal biosynthetic paths. One important factor in this connection is that the exogenous administration of an amino acid can sometimes induce the formation of degradatiye enzymes which are not formed when the organism has available only its endogenous supply of the same compound.

2) There has been excellent concordance among the results obtained in biosynthetic studies employing the mutant, the enzymatic, and the isotopic techniques. This fact increases our confidence that we are dealing with true metabolic intermediates rather than artefacts produced as a result of mutations or of cell disruption and fractionation. In particular, the strongest evidence in favor of a compound as an essential intermediate appears to be furnished by the demonstration that *a*) it is the respective product and substrate of a pair of enzymes in the wild-type cell, and *b*) the absence of either of these enzymes in a mutant cell results in a new growth-factor requirement (33, 34).

3) In contrast, the use of antimetabolites in inhibition analysis has not always furnished results in harmony with those obtained by independent methods. For example, the multiple requirement of aromatic auxotrophs of *E. coli* for tyrosine, phenylalanine, and tryptophan makes it clear that in this species these three compounds are not interconvertible but rather arise separately from common precursors. However, studies with antimetabolites have led various investigators to infer a number of different conversions among these aromatic amino acids in *E. coli*. A possible explanation of the discrepancy is provided by recent evidence (35, 36) that antimetabolites can interfere not only with the metabolism of normal cell constituents but also with their entry to the cell. The method of inhibition analysis must therefore expand its theoretical framework to take account of the possibilities that *a*) an inhibitor might function by excluding a required metabolite from the cell, and *b*) with an inhibitor that acts intracellularly, an antagonist might function by excluding the inhibitor from the cell rather than supplying the product of the blocked reaction.

4) Considerations of permeability have become increasingly important also in nutritional studies. Thus in the earlier biochemical applications of auxotrophic mutants there was a selection for those intermediates that were readily recognized through their activity as growth factors. But in the aromatic sequence, for example, and in histidine biosynthesis, the majority of the intermediates now known are devoid of nutritional activity. To be sure, nutritional (and isotopic) studies on intact cells have been very important, not only in revealing new intermediates but in validating the physiological significance of studies with extracted enzymes. It seems clear, however, that the latter approach will now be increasingly important in tracing out biosynthetic paths.

An interesting aspect of the permeability problem is the observation that certain intermediates that are devoid of nutritional activity because of impermeability (e.g., prephenic acid, compound Z1, and in certain strains DHQ) are nevertheless excreted in large quantities by mutants blocked after them. This fact suggests either one-way permeability, or an impermeability that is present under conditions of growth but not under the conditions that result in accumulation.

5) The limited comparative studies that have been carried out in bacteria and molds indicate great uniformity in the paths of amino acid biosynthesis. Indeed, the enzymes of aromatic biosynthesis that interconvert DHQ, DHS, and shikimate have been found not only in these classes of organisms, but also in higher plants. Thus far two well-documented exceptions to this uniformity have been established. *a*) Phenyl-

alanine can be converted to tyrosine by animal tissues and by certain microorganisms, but not by *E. coli*. *b*) Lysine is formed in *E. coli* by decarboxylation of a 7-carbon compound, α,α'-diaminopimelic acid, which is also a constituent of protein in this and certain other bacteria (37, 38). In Neurospora, on the other hand, in which DAP is not a protein constituent, lysine arises from a 6-carbon precursor, α-aminoadipic acid (39).

REFERENCES

1. Davis, B. D. *Advances Enzymol.* 16: 247, 1955.
2. McElroy, W. D. and B. Glass. Editors. *Amino Acid Metabolism.* Baltimore: Johns Hopkins Press, 1955.
3. Davis, B. D. In *Amino Acid Metabolism.* Baltimore: Johns Hopkins Press, 1955, p. 799.
4. Salamon, I. I. and B. D. Davis. *J. Am. Chem. Soc.* 75: 5567, 1953.
5. Davis, B. D. J. Biol. Chem. 191: 315, 1951.
6. Davis, B. D. and E. S. Mingioli. *J. Bact.* 66: 129, 1953.
7. Davis, B. D. *Science* 118: 251, 1953.
8. Weiss, U., C. Gilvarg, E. S. Mingioli and B. D. Davis. *Science* 119: 774, 1954.
9. Weiss, U., B. D. Davis and E. S. Mingioli. *J. Am. Chem. Soc.* 75: 5572, 1953.
10. Davis, B. D. and U. Weiss. *Arch. exptl. Path. Pharmakol.* 220: 1, 1953.
11. Gilvarg, C. Unpublished observations.
12. Mitsuhashi, S. and B. D. Davis. *Biochim. et Biophys. Acta* 15: 54, 1954.
13. Yaniv, H. and C. Gilvarg. *J. Biol. Chem.* 213: 787, 1955.
14. Mitsuhashi, S. and B. D. Davis. *Biochim. et Biophys. Acta* 15: 268, 1954.
15. Kalan, E. and B. D. Davis. Unpublished observations.
16. Srinivasan, P. R., H. T. Shigeura, M. Sprecher, D. B. Sprinson and B. D. Davis. In preparation.
17. Kalan, E., B. D. Davis, P. B. Srinivasan and D. B. Sprinson. In preparation.
18. Srinivasan, P. R., E. Kalan, D. B. Sprinson and B. D. Davis. In preparation.
19. Horecker, B. L., P. Z. Smyrniotis, H. H. Hiatt and P. A. Marks. *J. Biol. Chem.* 212: 827, 1955.
20. Srinivasan, P. R., M. Katagiri and D. B. Sprinson. Unpublished observations.
21. Ames, B. N. and H. K. Mitchell. *J. Am. Chem. Soc.* 74: 252, 1952.
22. Ames, B. N. In *Amino Acid Metabolism.* Baltimore: Johns Hopkins Press, 1955, p. 357.
23. Vogel, H. J., B. D. Davis and E. S. Mingioli. *J. Am. Chem. Soc.* 73: 1897, 1951.
24. Adams, E. *Federation Proc.* 14: 171, 1955.
25. Adelberg, E. A. *J. Am. Chem. Soc.* 76: 4241, 1954.
26. Strassman, M., A. J. Thomas, L. A. Locke and S. Weinhouse. *J. Am. Chem. Soc.* 76: 4241, 1954.
27. Strassman, M., A. J. Thomas and S. Weinhouse. *J. Am. Chem. Soc.* 77: 1261, 1955.
28. Abelson, P. H. *J. Biol. Chem.* 206: 335, 1954.
29. Strassman, M., L. A. Locke, A. J. Thomas and S. Weinhouse. *Science* 121: 303, 1955.
30. Vogel, H. J. and B. D. Davis. Unpublished observations.
31. Vogel, H. J. *Proc. Nat. Acad. Sci.* 39: 578, 1953.
32. Vogel, H. J. and B. D. Davis. *J. Am. Chem. Soc.* 74: 109, 1952.
33. Adelberg, E. A. *Bact. Rev.* 17: 253, 1953.
34. Davis, B. D. In *Perspectives and Horizons in Microbiology*, edited by S. A. Waksman. New Brunswick: Rutgers Univ. Press, 1955, p. 40.
35. Kihara, H. and E. E. Snell. *J. Biol. Chem.* 197: 791, 1952.
36. Mathieson, M. J. and D. G. Catcheside. Unpublished observations.
37. Dewey, D. L. and E. Work. *Nature* 169: 533, 1952.
38. Davis, B. D. *Nature* 169: 534, 1952.
39. Mitchell, H. K. and M. B. Houlahan. *J. Biol. Chem.* 174: 883, 1948.

HORMONAL CONTROL OF AMINO ACID METABOLISM[1]

JANE A. RUSSELL

From the Department of Biochemistry, Emory University, Emory University, Georgia

THE GENERAL metabolism of amino acids constitutes a system of the greatest biological importance; yet, of the factors which control and integrate the rates of these processes in the animal body we know very little. That controlling mechanisms of some sort must exist is obvious from the fact that nitrogen balance is the characteristic state of the normal adult individual. Furthermore, distinct aberrations in general amino acid metabolism or in protein balance are known to occur in many situations, and often these may be taken as evidence of some disturbance in the regulatory mechanisms. Of the processes known to participate in this regulation, some appear most probably to be endocrine in nature. Others, however, probably are not connected directly with any known hormonal function, while still others may represent combined manifestations of both hormonal and other agencies. Hence any discussion of the hormonal control of amino acid metabolism ought to begin at once with questions as to whether in fact any given case does represent direct intervention by endocrine mechanisms in the reactions concerned here.

One example of such a question relates to the loss of protein from the body which is characteristic of reaction to injury. This process is contingent on the presence of adrenocortical hormones; but according to current evidence, other mechanisms, not yet clearly identified except as consequences of the injury, are even more critical than hormonal activity in the production of the catabolic response (1–3). Another such question arises from the negative nitrogen balance seen in the absence of insulin. Here, we do not know whether the defect is the result of the lack of insulin per se, or whether it is secondary to the deficiency in carbohydrate metabolism and resultant lack of 'nitrogen sparing' effect, or whether it may result in whole or in part from a relative excess of other hormones opposing the action of insulin in this respect.

On the other side of the ledger, growth of any organ or tissue also may be considered to be evidence of an extraordinary imbalance between anabolic and catabolic processes. In some instances, growth of the body as a whole, or of certain organs only, may be influenced critically by one or another of the hormones; but is growth in these cases the result of the alteration in nitrogen metabolism apparently brought about by the hormone, or does retention of protein follow from changes induced by the hormone in some more intimate part of the cellular mechanisms?

These and many other questions concerning the control of amino acid metabolism cannot be answered at present. Instead, this paper will try only to outline some of the problems and to summarize the available evidence as to one facet of this field, that of demonstrable effects of the hormones on amino acids in the body. In this attempt, I shall confine myself mainly to evidence obtainable within at most a day or two after experimental intervention; for characteristic effects of most of the hormones on nitrogen metabolism can be seen within this time, and hence later effects are quite as likely as not to be secondary in nature. In the latter category must be included, for the present at least, most of the effects reported for excess or lack of some hormones on the content of various enzymes in the tissues. These effects have so far always been observed after at least several days of treatment; in many instances they have been obviously related to food intake or to altered metabolic rate; and in others they have been most readily interpreted as consequences, rather than causes, of changing rates of nitrogen metabolism.

PLASMA AMINO NITROGEN

In any study of the nature of hormonal controls in amino acid metabolism, the initial observations, one would expect, would concern the changes which might be induced by hormonal

[1] Supported in part by a grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council.

intervention in the concentrations of amino acids present in the plasma and in the tissues. These materials approximate, presumably, the amino nitrogen pool or pools, the rather small reservoirs of free amino acids from which components of the tissue proteins may be withdrawn or into which they may return, or from which the amino acids may be taken by deamination and subsequent operations. In the plasma and extracellular fluids, the amount of amino nitrogen is minute as compared to the total or to the protein nitrogen in the body, and even within the cells, in which the concentration of amino nitrogen is considerably greater than it is in plasma, the ratio of protein nitrogen to amino nitrogen is still about 100:1 or 50:1. The total amounts of free amino nitrogen in extracellular fluids in the body are small also in comparison with the daily turnover of nitrogen. In man and larger animals the latter is perhaps 10 times greater, and in the rat, 50 times the free amino nitrogen in circulation. In these circumstances, it might be expected that considerable changes in plasma and tissue amino nitrogen would occur in response to metabolic alterations induced by hormones or by other factors affecting nitrogen balance.

In fact, the plasma amino nitrogen concentration may be altered quite significantly in a short time by the administration of any of several different hormones, and a short selection of such data is presented in table 1. Unfortunately, some non-hormonal factors also have the same advantage, so that the effects of the hormones are neither highly reproducible nor entirely specific. As shown in table 1, glucose, when injected or fed in any quantity, will induce profound reductions in plasma amino nitrogen, and so will the intraperitoneal injection of any considerable amount of physiological saline. Handling of the animals or excitement may also induce variation. Hence, the reliability of any observations on plasma amino nitrogen must be assured by appropriate control measures as well as by replication of experiments. Additionally, if whole blood rather than plasma is analyzed, any appreciable change in hemoconcentration also must be avoided. These points have not always been appreciated, and so, unfortunately, some of the data in the literature are of questionable value in this connection.

In spite of these difficulties, however, clear evidence has been obtained that at least three hormones may bring about a rapid and significant reduction in the quantity of circulating amino

TABLE 1. EFFECTS OF VARIOUS TREATMENTS ON PLASMA AMINO N IN NORMAL RATS, 2–4 HR. AFTER TREATMENT*

		No. Observ.	Change in Amino N, mg %
A			
1	Saline, 0.2 ml/100 gm†	8	−0.3
2	Saline, 0.5 ml/100 gm†	4	−1.5
3	Glucose, 300 mg/100 gm‡	5	−2.8
B			
1	Insulin, 2 U/kg†	6	−2.6
	Insulin, 2 U/kg,† (not fasted)	7	−2.2
	Insulin, 2 U/kg,† (adrenal-demedullated, not fasted)	5	−2.0
2	Epinephrine, 0.05 mg/kg§	11	−2.5
3	Growth hormone, 1 mg/100 gm†	9	−2.8

* Fasted intact animals except where indicated. Initial plasma amino N concentration, 7–8 mg% (β naphthaquinone sulfonate or ninhydrin reactions). S.E. of differences, 0.2–0.4 mg%. † Intraperitoneal. ‡ Oral. § Intramuscular.

nitrogen. As shown in table 1, these are: insulin at rather high dosage (more is required for this effect than for lowering the blood glucose); epinephrine in moderate dosage; and growth hormone, also in rather large amount. Additionally, the plasma amino nitrogen may sometimes be altered by adrenocortical steroids or adrenocorticotrophic hormone. Most frequently an elevation has been reported, but occasionally a short period of depression in amino nitrogen level has been seen instead.

On the other hand, operative removal of any of several endocrine organs does not seem to induce any characteristic alterations in plasma amino nitrogen (table 2). In my experience, hypophysectomized rats have usually displayed normal levels, although this may be elevated if the animal is not in very good condition. In alloxan treated rats, some weeks after induction of diabetes, the amino nitrogen levels tend to be somewhat elevated, but obviously many of these values were in the normal range. Evidently, although alterations in plasma amino nitrogen may reflect acute changes in metabolic pattern, the effects of chronic deficiency of hormonal regulatory factors must be rather readily subject to physiological compensation.

The similar reductions in plasma amino acids brought about by insulin, epinephrine, and growth hormone have led to the question whether any one of these hormones may in fact be the primary agent, the others acting only through its mediation, or if some or all of them may act

independently in this respect. Some years ago, David and Van Winkel (4) reported that in adrenodemedullated rabbits insulin failed to exhibit its characteristic action on the blood amino nitrogen, and hence they suggested that epinephrine was the primary actor. It seems quite likely that epinephrine may indeed play a part in the insulin effect when hypoglycemia has been induced. However, in eviscerated rats which had been adrenodemedullated prior to experiment, and in which glucose was given to prevent hypoglycemia, insulin was able to reduce the rate of increase in blood amino nitrogen just as well as it did in animals with intact adrenals (5); and, furthermore, the action of insulin in such preparations is independent of the blood glucose level (6). More recently, we have observed that insulin will depress the blood amino nitrogen in fed rats in which the blood glucose does not usually fall low enough to stimulate epinephrine secretion, and this occurs both in normal and in adrenodemedullated animals (table 1). Hence, it appears that insulin may affect the plasma amino nitrogen independently of the intervention of epinephrine secretion. The converse possibility, that epinephrine might act through the mediation of insulin secretion, also has been disproved by observations made by several investigators that epinephrine brings about the usual reduction in blood amino nitrogen in diabetic animals.

Whether or not the effects of growth hormone on blood amino nitrogen might be related to those of insulin or of epinephrine has also been considered. From the observations of Milman and Russell in rats (7) and of Milman, de Moor and Lukens in cats (8), the blood amino nitrogen of diabetic animals appears to be as responsive to growth hormone as that of normal animals. Recently, Luck and his colleagues suggested that growth hormone might act in this respect through

TABLE 2. PLASMA AMINO N CONCENTRATIONS IN RATS* (β NAPHTHAQUINONE SULFONATE REACTION)

		No. Observ.	Amino N, mg %
1	Normal	63	7.5 ± 0.1†
	Normal, not fasted	13	8.3 ± 0.2
2	Hypophysectomized	15	7.9 ± 0.2
3	Adrenalectomized (salt-treated)	12	7.2 ± 0.2
4	Adrenodemedullated	18	7.9 ± 0.2
5	Diabetic (alloxan)	11	8.8 ± 0.3

* Fasting animals except where indicated.
† S.E.

TABLE 3. EFFECTS OF GROWTH HORMONE OR EPINEPHRINE ON PLASMA AMINO N IN ADRENALECTOMIZED RATS

	Growth Hormone	Epinephrine
Normal rats	−2.5 ± 0.2(4)	−2.5 ± 0.3(11)
Adrenalect.	−2.1 ± 0.3(8)	−2.1 ± 0.2(8)
Hypophysect.		−1.9 ± 0.2(11)

adrenal-medullary or possibly through adrenocortical secretion, for they did not observe what they considered to be significant depression of the blood amino nitrogen in adrenalectomized rats given growth hormone (9). However, we have seen such an effect repeatedly, and an example is shown in table 3. From these observations, it appears that growth hormone may act independently of either islet or adrenal secretions.

On the other hand, we once considered the possibility that epinephrine might bring about depression of the amino nitrogen levels by inducing secondary secretion of one or more of the pituitary hormones; but as shown also in table 3, this idea too was proved untenable. A similar observation has been made also in hypophysectomized cats by Milman *et al.* (8).

The remaining permutation of possible interrelations here, that insulin would require for its effect the secretion of pituitary growth hormone, has not been completely explored, for in the absence of the hypophysis only small amounts of insulin may be given. In hypophysectomized rats, we have observed after insulin reductions in the blood amino nitrogen as great as 50%, but since the animals were by that time in hypoglycemic shock, it is not certain that the blood samples obtained were representative of the circulating fluid. In cats, the usual fall in blood amino nitrogen after insulin was reported by Milman *et al.* (8).

TISSUE AMINO NITROGEN AND NITROGEN BALANCE

The observations just described do not by themselves permit any conclusions concerning the mechanisms by which these changes in plasma amino nitrogen may be brought about; but combined with other observations they must have meaning. Moreover, they must be explicable in terms of any hypotheses concerning the hormonal control of amino acid metabolism. As a first approach to rationalizing these effects, we may inquire whether concurrently with the drop in plasma amino nitrogen any changes occur in the concentrations of free amino nitrogen in the liver

and in other tissues or in the rate of nitrogen catabolism. Unfortunately, but few studies have been made on the acute effects of the hormones on the nonprotein nitrogen of the tissues, and none are available in which the changes in plasma amino nitrogen, tissues nitrogen, and rates of urea formation have been observed simultaneously. In table 4 is summarized what is known of these effects. Those on growth hormone are the best attested and most clear-cut. As is well known, a positive nitrogen balance is seen after growth hormone, and nitrogen retention may be demonstrated readily in short-term experiments if exogenous amino acids are given. Shaffer and Lee, employing in 1935 a globulin fraction from the pituitary, first reported an acute reduction in the concentration of amino nitrogen in the tissues (10). More recently, we have attempted to verify this observation using purified growth hormone, and have succeeded in doing so in part. As shown in table 5, the effects obtained in liver are definite with respect both to amino and to amide nitrogen. In muscle, possibly the same changes may have occurred, although here the amounts of amino nitrogen, which includes such things as carnosine, are larger and more variable and so small changes are more difficult to detect. In any case, these observations do afford one conclusion. This is that the acute anabolic effect of growth hormone is not probably the result of inhibition of amino acid catabolism per se; for in the latter event, the amino nitrogen of the liver would have been expected to be increased, especially when exogenous amino acids were given. Some further aspects of the acute effects of growth hormone will be considered later in this paper.

Whether insulin or epinephrine may induce other changes besides depression of the plasma amino nitrogen is not so clear. In the fasting subject, or even in the fed animal if the dose of hormone is large, insulin may increase the rate of nitrogen catabolism considerably. This was reported by Kiech and Luck (11) and by others at an early date and it has been observed more recently also by Ingle (12) and by Engel (13). On the other hand, of course, insulin with carbohydrate will restore the nitrogen balance in diabetic subjects, and such treatment over a period of time has even been reported to allow some storage of nitrogen in the hypophysectomized animal rat (14). Thus, whether insulin has an anabolic or a catabolic effect appears to depend on the circumstances of the observation.

TABLE 4. EFFECTS OF SOME HORMONES ON AMINO N CONTENT AND CATABOLISM

	Change in Free Amino N			N Excretion	
	Plasma	*Liver*	*Muscle*	*Fasting*	*Fed CHO*
Growth Hormone	−	−	−	−	− or 0
Insulin	−	− ?	−	+	+ or −
Epinephrine	−	− ?	− ?	+	. . . .
Adrenocortical hormone	±	±	±	+	+

With epinephrine, also, increased catabolism of nitrogen has been reported (3, 15), although it must be noted that the doses required for this effect are rather large. The loss of nitrogen after either hypoglycemic doses of insulin or after epinephrine, like that after trauma or after many noxious substances, has been found by Engel to be contingent on the presence of adrenocortical hormone and to be hastened and augmented by the addition of this agent. Also in Engel's experiments, the acute effects of both hormones on nitrogen catabolism were preventable with glucose in quantity. Hence, the immediate actions of either or both hormones in nitrogen balance may be of a non-specific character. Understandably, the evidence with respect to effects of these hormones on tissue constituents is conflicting. Luck and Morse (16) observed reduction in the amino nitrogen content of both liver and muscle after either insulin or epinephrine, but this was not confirmed by Friedberg and Greenberg (17) in either instance. More recently, however, Bollman *et al.* (18) reported a reduction in the amino nitrogen of muscle in hepatectomized dogs given insulin. Obviously, we are still uncertain as to the actual nature of the changes induced by these two hormones in the nitrogenous constituents of the body tissues. The possibility that some of the actions of the hormones on nitrogen catabolism may be non-specific or 'stress' effects does not of course, obviate the problems of explaining the mechanism of these effects or of relating the changes in nitrogen balance to the changes in plasma amino nitrogen which may occur concurrently.

Of the group of hormones which can bring about acute alterations in nitrogen metabolism, those of the adrenal cortex remain to be mentioned in this connection. As is well known, the cortical steroids oxygenated at C-11, either administered as such or secreted under the influence of ACTH, tend to induce loss of nitrogen, and

TABLE 5. EFFECT OF GROWTH HORMONE ON AMINO N IN TISSUE*

	Amino N, mg %		Amide N + NH_3 mg %	
	Control	GH effect	Control	GH effect
Plasma				
Fasting	8.8	−2.0 ± .18†		
Casein hydrol.‡	10.2	−1.8 ± .42†		
Liver				
Fasting	55	−8.3 ± 1.7†	17	−4.6 ± 0.7†
Casein hydrol.‡	54	−5.6 ± 1.8†	22	−6.0 ± 2.6†
Gastroc. muscle				
Fasting	75	−0.3 ± 1.4	17	−0.4 ± 0.8
Casein hydrol.‡	77	−3.6 ± 1.5†	18	−0.3 ± 2.5
Heart				
Fasting	98	−4.8 ± 1.8†	21	−6.4 ± 2.0†
Casein hydrol.‡	100	−1.0 ± 2.0	26	−5.5 ± 1.7†

* OSTASHEVER AND RUSSELL, unpublished observations. Growth hormone, 1 mg/100 gm, was given i.p. 4 hr. before analysis. Each group comprised 7–10 observations.

† *P* of chance difference 0.05 or less.

‡ Casein hydrolysate (Amigen), 10 mg N/100 gm, was given i.v. 1½ hr. before analysis.

this effect may be seen within a few hours. Reports on the effects of these hormones on the amino acids of blood and tissues have not been illuminating. Usually the plasma amino nitrogen has been reported to be increased, but sometimes there has been no change, or occasionally a temporary decrease has been seen, and the few data available on the amino nitrogen of the tissues is similarly conflicting (17, 19, 20). Since the catabolic response to cortical hormone can be much influenced by nutritional state and by other factors, as the presence of noxious stimuli, and since the observations on plasma and tissue constituents were not made simultaneously with those on nitrogen catabolism, this confusion is not surprising.

ENDOGENOUS AND EXOGENOUS AMINO ACID METABOLISM

Obviously, the attempt to correlate the acute effects of the several hormones on the nitrogenous constituents of the body described above has led to few conclusions. This is so probably not because the task is inherently impossible, but because so few sets of observations have been designed with this end in view. Of recent years, however, these and related problems have been approached by means of other experiments of somewhat more dynamic character. These have met with somewhat greater success, but even so, the general lack of simultaneous or parallel observations on different aspects of the problem has sometimes made interpretation difficult. These experiments fall into three general categories. In one, measurements have been made of the rate of release of amino nitrogen from the peripheral tissues of eviscerated or hepatectomized animals. This affords a measure of at least the net rate of protein breakdown in the tissues, independent of rates of amino acid catabolism per se. In another type of experiment, the disposition of exogenous mixed amino acids is observed in terms of rates of urea formation or sometimes in terms of the disappearance of amino nitrogen from blood and tissues. This has been most commonly carried out in nephrectomized rats, in which rather precise measurements are possible over short periods of time. The third general category includes studies made with isotopic amino acids, to which reference will be made later. The results reported in respect to the actions of the hormones are summarized in tables 6 and 9.

With these techniques, some additional facts have emerged. The most impressive of these perhaps is the effect of insulin in the eviscerate animal. Here, as first demonstrated by Mirsky, the steady loss of amino acids from the peripheral tissues, characteristic of this preparation, is much diminished in rate by insulin (5, 21), or even, if the rate of increase in plasma amino nitrogen is not rapid, it may be suppressed almost entirely by this hormone (6). The amounts of insulin required are moderately large, and so some glucose must always be given with the insulin; but glucose alone has no such effect, nor is the amount of amino nitrogen released related to the blood glucose level when insulin is present. Thus, in the presence of an indeterminate amount of carbohydrate, insulin can affect the nitrogen balance within the tissues in a relatively direct fashion, and the processes of deamination or of subsequent gluconeogenesis need not be concerned in this activity.

TABLE 6. EFFECTS OF SOME HORMONES ON AMINO ACID METABOLISM

	Amino N Release in Eviscerate Animals	Catabolism of Exogenous Amino Acids
Growth hormone	−*	−
Insulin	−	0
Epinephrine	. . . .	0
Adrenocortical hormone	+	0

* In presence of insulin.

Other hormones besides insulin can affect the rate of release of amino acids from the tissues. In many attempts to demonstrate growth hormone activity in the eviscerate animal, my colleagues and I have never been able to obtain convincing evidence of alteration in the rate of accumulation of amino nitrogen in the blood when this hormone was given alone. However, an effect of growth hormone in the absence of the liver and viscera can be observed in two different circumstances; one, if insulin is given also, pituitary extract or purified growth hormone will further depress the rate of release of amino nitrogen from the tissues (5, 22), and secondly, if exogenous amino acids are given, these are removed from the blood stream to a greater extent in growth hormone treated animals than in the controls (22). Some representative data are shown in table 7. Insulin alone has a similar effect, and if the two hormones are given concurrently, the action is approximately additive. A somewhat similar requirement for the presence of insulin in the action of growth hormone in respect to nitrogen balance in rats has been reported by Milman *et al.* (8). The meaning of these relationships is still obscure. From these and other observations, it appears that the acute effect of growth hormone on nitrogen retention is best demonstrated in the presence of a plethora of amino acids. Perhaps then the effect of insulin may be to bring about somewhat the same state of affairs within the cells and so make possible the further retention of amino acids by growth hormone. This it might do by enabling indirectly the retention of amino acids within the cells, or it could perhaps promote the synthesis of lower peptides, or other means could be imagined; but as yet there is no evidence in this point.

Adrenocortical hormone has been shown by Bondy and by Ingle to enhance the rate of release of amino acids from the tissues of eviscerate animals (23–25). This is an important observation, for it indicates that neither the process of deamination of amino acids nor that of gluconeogenesis is necessarily a primary site of action of this hormone.

The possible effect of epinephrine on the release of amino nitrogen from the tissues does not appear to have been reported. However, among observations made some years ago by Dr. Frame and myself (5), we noted that adrenodemedullated rats did not differ from the normal in respect to release of amino acids after evisceration. Since other evidence indicates massive discharge of epinephrine from intact adrenal glands during this operation, one would guess that administered epinephrine similarly would have but little effect; but more conclusive observations on this point are needed. If this is true, it would mean most probably that the action of epinephrine was primarily upon the rate of removal of amino acids by the liver.

TABLE 7. EFFECTS OF GROWTH HORMONE AND INSULIN ON PLASMA AMINO N IN EVISCERATED RATS (INCREASE, MG %)

A. Release of amino N in 3 hr. period between 2 and 5 hr. post-op.	No insulin	Insulin. 1 u/kg, s.c.
Without GH	+13.8 ± 0.5* (11)	+6.2 ± 0.5 (7)
GH (0.5 mg/100 gm, 1 hr. pre-op. and ½ hr. post-op.)	+13.7 ± 0.4 (4)	+3.2† ± 0.5 (12)
B. Added amino acids: Incr. in plasma amino N 30 min. after injection (1–1½ hr. post-op.)	Saline control	*Casein hydrol.* 4.5 mg N/100 gm, i.v.
Untreated	+1.4 ± 0.2 (10)	+7.1 ± 0.2 (9)
GH (0.5 mg, 2 and 5 hr. pre-op.)	+1.1 ± 0.2 (6)	+5.6† ± 0.3 (7)

* S.E. † P of chance difference < 0.01.
Number of observations in parentheses.

The demonstration of alterations in the rate of accumulation of amino acids in the blood after removal of the liver is important in that it may allow us to distinguish between effects on amino acid metabolism per se and those having to do with the protein balance within the tissues; but they do not indicate whether the latter are concerned with the synthesis of protein or its breakdown. One type of experiment which may afford information on this point is observation of the disposition of a considerable amount of readily utilizable amino acids. If the hormonal effect in question were solely upon the release of amino acids from tissue proteins, the characteristic effect ought not to be altered materially whatever the quantity or quality of amino acids presented. On the other hand, if the synthesis of protein were either to be augmented or to be seriously depressed in an acute experiment, one might expect to see exaggeration of the changes in nitrogen balance in the presence of complete mixtures of amino acids. In the case of growth hormone, this is what happens. In short term experiments in the fasting animal, only small and often insignificant effects on nitrogen balance may be seen. However, when amino acids are provided in the form of casein hydrolysate, the rate of

TABLE 8. EFFECTS OF GROWTH HORMONE ON UREA FORMATION IN NEPHRECTOMIZED RATS (INCREASE IN BLOOD UREA N IN 2 HR.*, MG %)

	Saline Control	Casein Hydrol. (13.5 mg N/100 gm i.v.)
A. Fasting animals		
1. Untreated	+6.5 ± 1.4 (4)	+22.2 ± 0.6 (10)
2. GH (1-2 hr. before observation)		
0.03 mg/100 gm		+19.3 ± 0.5 (4)
0.10 mg/100 gm		+16.5 ± 0.7 (5)
1.0 mg/100 gm	+4.6 ± 0.8 (4)	+14.5 ± 0.5 (7)
B. With glucose		
1. Glucose s.c. (50–100 mg/100 gm) 1 hr. before observation)		+17.9 ± 0.3 (8)
2. GH (1 mg/100 gm) with glucose as above		+13.4 ± 0.3 (8)

* Observations begun 3 hr. after nephrectomy with blood samples taken immediately before injections of amino acids or saline.

urea formation immediately following their administration is notably diminished when the animals are pretreated shortly before with growth hormone (table 8). This effect is quite reproducible and affords the basis of a method assay of growth hormone activity (26, 27).

In similar circumstances, none of the other hormones which we have discussed has been observed to have any effect. In Engel's work, the action of cortical hormone on nitrogen catabolism appeared even to be inhibited somewhat by exogenous amino acids, for no greater increase in urea formation was seen when both the amino acid mixture and cortical hormone or ACTH were given together than was obtained when either was given separately (28). In substantial agreement with this observation is the work of Silber and Porter (29) who found that the nitrogen loss induced by large doses of cortisone was independent of the protein intake. Neither insulin nor epinephrine, in the moderate doses which we have used (30) nor in the larger amounts employed by Engel (2), has had any effect on the rate of urea formation following amino acid mixtures. In these experiments, no glucose was given with the insulin since the object was to study the effects of hypoglycemia. However, in the intact animal glucose alone will inhibit urea formation after the administration of amino acid mixtures (table 8), and of course the similar nitrogen sparing effect of carbohydrate in the fed animal is well known. It is possible that this represents the anabolic effect of the animal's own insulin combined with carbohydrate; but the critical experiment, the observation in diabetic animals of the effects of glucose and insulin alone and in combination on the metabolism of administered amino acids, has not been performed.

From the results obtained in the latter group of experiments, where the rate of urea formation was used as the indicator of the fate of the amino acids given, it is of course not possible to distinguish between effects of the hormone on retention of the amino acids as protein and those on the rates of deamination or on urea formation per se. For the interpretation of the action of growth hormone, for example, we must refer to other changes which accompany this effect. Here, as noted previously, the concentration of amino and of amide nitrogen in the liver was reduced after growth hormone whether or not exogenous amino acids were given; and hence it is not likely that the diminution in rate of urea formation after growth hormone was to any great extent the consequence of a reduction in the rate of catabolism of the amino acids themselves. Similarly, the rate of removal of amino acids from the blood has been found to be augmented, if anything, after growth hormone has been given, and it will be recalled also that small but significant effects of the hormone could be obtained in the eviscerate preparation. Hence, it is probable that the action of growth hormone here was to augment the uptake of amino acids into the tissues, presumably as protein, and that the reduction in urea formation was an inverse reflection of this change.

METABOLISM OF ISOTOPIC AMINO ACIDS

Finally, we may consider the attempts which have been made in recent years to investigate the actions of the hormones on nitrogen metabolism through the use of isotopic tracer techniques. A summary of relevant observations which have appeared in the literature is presented in table 9. In this work, a considerable group of experiments are those of Hoberman (31) and of others, who have utilized variants of the method first introduced by Sprinson and Rittenberg (32)—namely, measurement of the excretion of isotopic nitrogen after the injection of labeled glycine. The original kinetic analysis of the excretion curves, based on what must be a gross oversimplification of the system of protein metabolism in the whole animal, probably cannot be considered accurate; for as San Pietro and Rittenberg (33) have pointed out,

the urea pool must be distinguished from the amino nitrogen pool, and as will be indicated later, it is probable also that there is more than one amino nitrogen pool. Further, the assumption of first order reaction rates, made by some other investigators, cannot be justified on present evidence, and so comparisons of rates of excretion of isotope in terms of fractions of apparent pool size can mean very little. However, the observations which have been made do indicate some significant alterations in the metabolism of isotopic amino acids under the influence of some of the hormones, and these must be taken into account in deriving concepts of the endocrine control of nitrogen metabolism.

In fasting hypophysectomized rats, Hoberman observed that the proportion of the isotope given which was excreted in 48 hours was increased above normal by about ⅓. During the same time, the total nitrogen excretion was unchanged. Apparently, then, the uptake of the labeled amino acid into body protein was diminished in this condition. If the concentration of isotope in the excreted nitrogen may be taken as representative of that in a homogeneous pool of amino acids undergoing turnover by replacement from body tissues and catabolism, then it would follow, since the concentration of isotope in the urine nitrogen here was high, that the return of unlabeled nitrogen from the body tissues also had been diminished. On the other hand, it may be argued that the isotope was excreted in higher concentration simply because it was not taken up into tissue proteins, or alternatively, but much less likely, that the labeled amino acid had been catabolized at a more rapid rate and so less was left for incorporation into protein. Only analysis of the isotope concentration in the amino nitrogen and other forms in the liver and elsewhere might decide among these possible interpretations.

Similar effects were observed in thyroidectomized animals, and so it is possible that part of the changes in turnover of amino acids indicated in the hypophysectomized animal were due to altered metabolic rate. On the other hand, however, growth hormone, given to fasting hypophysectomized, thyroidectomized or adrenalectomized rats, brought about significant reduction in the excretion of labeled nitrogen and also of total nitrogen in proportionate degree. This may be interpreted to mean that the synthesis of protein had been increased by growth hormone, and that concomitantly little change had occurred in the rate of catabolism of body protein. A considerable reduction in the rate of excretion of labeled nitrogen and also of total nitrogen in fed dogs treated with growth hormone also has been observed by Bartlett and Gaebler (34). The few observations available on the actual incorporation of labeled amino acids into protein indicate that growth hormone treatment may have accelerated the synthesis of protein (35, 36). These observations thus are in essential agreement with the conclusions drawn from other types of experimental observations, that is, that growth hormone favors the synthesis of protein from amino acids, but they have as yet given no greater insight into the mechanisms affected by growth hormone.

TABLE 9. HORMONAL EFFECTS ON METABOLISM OF ISOTOPIC AMINO ACIDS

	N^{15} Excretion*	Dilution of Excreted N^{15} by Unlabeled N†	Incorporation‡ of N^{15}, C^{14}, or S^{35}
Hypophysectomized	+	−	−
Adrenalectomized	0	−	−liver; other?
Thyroidectomized	+	−	.....
Diabetic (alloxan or depanc.)	+	+	−
Adrenalect.-diabetic (cf. diabetic)	−	−	.....
Growth hormone	−	0	+
Adrenocort. hormone	+	+	+ liver ± muscle
Insulin	....	....	+ muscle

* After isotopic glycine (31, 34, 40).
† In fasting animals (31).
‡ From amino acids into tissue protein (35–39, 41, 43).

In the tissues of diabetic animals, the rate of incorporation of amino acids labeled with C^{14} or S^{35} has been reported by Krahl (37) and by Forker *et al.* (38) to be quite low. This rate could be restored to normal by pretreatment with insulin or, in *in vitro* experiments, by addition of insulin to the medium (37, 39). These observations suggest that insulin directly or indirectly enhances the rate of synthesis of protein; but the evidence as yet available does not allow much further interpretation.

With respect to the action of adrenocortical hormone, some additional information has perhaps been added. Here several sets of observations agree that when adrenocortical activity is increased, the excretion of labeled amino nitrogen as urea also is increased markedly (31, 40, 41). This has been taken by some to mean that the synthesis of protein must have been diminished; but the data of Hoberman (31) offer an alternative interpretation. Here, in the absence of exogenous amino acids, the excretion of unlabeled

nitrogen was increased to an even greater degree than was that of the isotope. Hence, to account for the observed dilution of urinary isotope, it seems quite probable that the breakdown of body protein must have been increased considerably also. According to the conventional calculation, these data indicated no change in the rate of synthesis of protein from the labeled pool. It may be noted that in alloxan diabetic rats similar changes were seen in the excretion of labeled and unlabeled nitrogen; and that these were nearly completely reversed by subsequent adrenalectomy.

In accord with the view that protein breakdown was increased, rather than synthesis diminished, are the later data of Clark (41). Here, in fed rats treated with cortisone, the excretion of isotope and of nitrogen were increased about proportionately. At the same time, the incorporation of isotope into the proteins of the empty carcass was perhaps diminished slightly, whereas that into the proteins of the liver and of the plasma was increased considerably, rather than diminished as would have been expected. Now if under the influence of excess cortical hormone increased quantities of amino acids are released from the peripheral tissues, these must be funneled through the liver. Here they would mix with part of the administered labeled amino acid; and then their joint fate would depend on the direction of metabolism in that organ. With enhanced supplies of amino acids, an increase in protein synthesis could occur in the liver, and such an effect of cortical hormone not only has been seen in the experiments of Clark, just described, but also demonstrated by Roberts in the regenerating liver and in isolated liver slices (42). Simultaneously, the catabolism of amino acids has been increased also in most instances, as evidenced by the loss of nitrogen from the body. Hence, the isotopic nitrogen may in effect be washed out of the body by the ingress of amino acids to the liver from other areas. The increased excretion of isotopic nitrogen in animals treated with excess cortical hormone would then be the result not of any primary diminution in protein synthesis, but instead would be due mainly to the movement of amino acids from the body proteins towards eventual destruction in the liver.

CONCLUSION

This last example of endocrine influence on nitrogen metabolism illustrates well the complexities of this field. From this and other evidence, it is obvious that the economy of amino acids in the intact animal cannot be represented by a simple homogeneous system, and that no one set of observations on a single phase is completely interpretable by itself. Rather, the evidence concerning the mechanisms of control of the many processes concerned must be made from multivariate analyses, in diverse experimental situations, and this work has only just begun. At present we can say, perhaps, that of the three principal hormones known to be important in this connection: insulin, cortical hormones, and growth hormone, none appears to be concerned primarily or exclusively with the catabolism of the amino acids per se or with gluconeogenesis from the fragments; but instead they all appear to influence some aspect of the synthesis or breakdown of proteins within most or many tissues. As yet, the nature of these regulative mechanisms is completely unknown.

REFERENCES

1. Ingle, D. J., E. O. Ward and M. H. Kuizenga. *Am. J. Physiol.* 149: 510, 1947.
2. Ingle, D. J. *J. Endocrinol.* 8: xxiii, 1952.
3. Engel, F. L. *Endocrinology* 50: 462, 1952.
4. Davis, B. L. and W. Van Winkle. *J. Biol. Chem.* 104: 207, 1934.
5. Frame, E. G. and J. A. Russell. *Endocrinology* 39: 420, 1946.
6. Ingle, D. J., M. C. Prestrud and J. E. Nezamis. *Am. J. Physiol.* 150: 682, 1947.
7. Milman, A. E. and J. A. Russell. *Endocrinology* 47: 114, 1950.
8. Milman, A. E., P. DeMoor and F. D. W. Lukens. *Am. J. Physiol.* 166: 354, 1951.
9. Luck, J. M., A. C. Griffin, G. Boer and M. Wilson. *J. Biol. Chem.* 206: 767, 1954.
10. Schaffer, N. K. and M. Lee. *J. Biol. Chem.* 108: 355, 1935.
11. Kiech, V. C. and J. M. Luck. *J. Biol. Chem.* 78: 257, 1928.
12. Ingle, D. J., J. S. Evans and R. Sheppard. *Endocrinology* 35: 370, 1944.
13. Engel, F. L. *Endocrinology* 45: 170, 1949.
14. Salter, J. and C. H. Best. *Brit. Med. J.* (2), 353, 1953.
15. Noble, R. L. and C. G. Toby. *J. Endocrinol.* 5: 303, 1948.
16. Luck, J. M. and S. W. Morse. *Biochem. J.* 27: 1648, 1933.
17. Friedberg, F. and D. M. Greenberg. *J. Biol. Chem.* 168: 405, 1947.
18. Bollman, J. L., E. V. Flock, J. H. Grindlay, F. C. Mann and M. A. Block. *Am. J. Physiol.* 174: 467, 1953.
19. Lee, N. D. and R. H. Williams. *Proc. Soc. Exper. Biol. & Med.* 79: 669, 1952.

20. DUBREUIL, R. AND P. S. TAMIRAS. *Am. J. Physiol.* 174: 20, 1953.
21. MIRSKY, I. A. *Am. J. Physiol.* 124: 569, 1938.
22. RUSSELL, J. A. *Protein Metabolism, Hormones and Growth.* New Brunswick: Rutgers Univ. Press, 1953, p. 46.
23. BONDY, P. K. *Endocrinology* 45: 605, 1949.
24. INGLE, D. J., M. C. PRESTRUD AND J. E. NEZAMIS. *Proc. Soc. Exper. Biol. & Med.* 67: 321, 1948; 75: 801, 1950.
25. BONDY, P. K., D. J. INGLE AND R. C. MEEKS. *Endocrinology* 55: 354, 1954.
26. RUSSELL, J. A. *Endocrinology* 49: 99, 1951.
27. RUSSELL, J. A. In: *Hypophyseal Growth Hormone, Nature, and Actions,* edited by SMITH, GAEBLER AND LONG. New York: McGraw-Hill, 1955, p. 17.
28. ENGEL, F. L., S. SCHILLER AND I. E. PENTZ. *Endocrinology* 44: 458, 1949.
29. SILBER, R. H. AND C. C. PORTER. *Endocrinology* 52: 518, 1953.
30. RUSSELL, J. A. AND M. CAPIELLO. *Endocrinology* 44: 127, 1949.
31. HOBERMAN, H. D. *Yale J. Biol. and Med.* 22: 341, 1950.
32. SPRINSON, D. B. AND D. RITTENBERG. *J. Biol. Chem.* 180: 715, 1949.
33. SAN PIETRO, A. AND D. RITTENBERG. *J. Biol. Chem.* 201: 457, 1953.
34. BARTLETT, P. D. AND O. H. GAEBLER. *J. Biol. Chem.* 196: 1, 1952.
35. FRIEDBERG, F. AND D. M. GREENBERG. *Arch. Biochem.* 17: 193, 1948.
36. LEE, N. D. AND R. H. WILLIAMS. *Endocrinology* 51: 451, 1952.
37. KRAHL, M. E. *J. Biol. Chem.* 200: 99, 1953.
38. FORKER, L. L., I. L. CHAIKOFF, C. ENTENMAN AND H. TARVER. *J. Biol. Chem.* 188: 37, 1951.
39. SINEX, F. M., J. MACMULLEN AND H. B. HASTINGS. *J. Biol. Chem.* 198: 615, 1952.
40. PARSON, W., K. R. KRISPELL AND A. EBBERT. *J. Clin. Invest.* 31: 548, 1952.
41. CLARK, I. *J. Biol. Chem.* 200: 69, 1953.
42. ROBERTS, S. *J. Biol. Chem.* 200: 77, 1953.
43. MARSHALL, L. M. AND F. FRIEDBERG. *Endocrinology* 48: 113, 1951.

DISCUSSION[1]

DWIGHT J. INGLE

From the Ben May Laboratory for Cancer Research, The University of Chicago, Chicago, Illinois

I WISH to emphasize a few general points concerning hormones and metabolism and to present new data of my own which relate to the studies of Dr. Russell upon the eviscerate rat.

Most hormones affect the metabolism of proteins under conditions of hormonal insufficiency or excess. It is common to describe the endocrine glands as regulating metabolism by changes in the amount of hormone released from the gland, but the relationship of the action of a hormone to the metabolic processes which it affects is frequently more complex. The titer of hormone in the body fluids is determined by the rate of its destruction as well as by the rate of secretion, so that the quantitative effect of the hormone can change when either the rate of secretion or intake of exogenous hormone is constant. Moreover, it seems probable that the efficiency of hormonal utilization is a variable. Many non-hormonal factors affect the rates of metabolic processes, in some instances to a more important extent than do the hormones. Hormones may support or permit metabolic responses by normalizing the functions of the tissues involved in the metabolic response. This is called a 'permissive' role of hormones and has been discussed in detail elsewhere (1). It seems reasonable to regard the regulation of metabolic processes as highly complex, involving a *Gestalt* of causal relationships, hormonal and non-hormonal, in which it is not wise to identify single principles as all-important.

My own studies support the more extensive and penetrating studies of Dr. Russell in showing that at least several of the hormones affect the level of plasma amino acids in the eviscerate rat, thereby proving that the liver and gut are not the sole sites of action of these hormones. Indeed, we may ask, "For which hormones is the liver an important target organ?" The glycogenolytic actions of epinephrine and of glucagon are readily demonstrable in the isolated liver. Acceptable evidence for the direct action of other hormones upon liver is scanty. Perhaps Dr. Miller will not mind the expression of a hope that he will include tests of hormones in his elegant studies on isolated liver.

In addition to its action upon carbohydrate metabolism, insulin affects the metabolism of proteins. I am interested in the nature of the relationship between these two general effects. Dr. Russell has reported that insulin will suppress the rise in plasma amino acids following evisceration. Insulin also has a striking effect upon the glucose tolerance of the eviscerate animal. Stimulation of muscle

[1] This work was supported by grants from The American Cancer Society as recommended by the Committee on Growth, and from the Public Health Service.

TABLE 1. EFFECT OF MUSCLE WORK ON LEVEL OF BLOOD GLUCOSE AND OF PLASMA AMINO ACIDS IN FUNCTIONALLY EVISCERATED RATS (AV. AND S.E.)

	Time Hr.	Load* Glucose	No. Rats	Plasma Amino Acids†	Blood Glucose†
Normal	0	0	6	5.34 ± 0.3	127 ± 7.63
Eviscerated					
No work	5	50	3	16.24	531
Work 1 leg	5	50	3	20.94	123
No work	4	160	3	15.04	985
Work 2 legs	4	160	3	16.73	372
No work	3	50	6	10.89 ± 0.3118	405 ± 10.43
Work 1 leg	3	50	6	12.00 ± 0.268	76 ± 14.61

* Mg/100 gm rat/hr.
† Final concentration, mg %.

TABLE 2. COMPARISON OF EFFECTS OF MUSCLE WORK AND OF INSULIN ON LEVELS OF BLOOD GLUCOSE AND OF AMINO ACIDS IN FUNCTIONALLY EVISCERATED RATS DURING 3 HOURS (AV. AND S.E.)

	Load Glucose*	Insulin†	No. Rats	Plasma Amino Acids‡	Blood Glucose‡
Normal	0	0	11	5.8 ± 0.21	132 ± 5.22
Eviscerated					
No work	100	0	12	11.1 ± 0.48	586 ± 23.3
No work	100	16	12	5.0 ± 0.23	241 ± 21.1
Work 2 legs	100	0	12	11.0 ± 0.31	99 ± 9.3

* Mg/100/hr. † u/day/rat. ‡ Final concentration, mg %.

has an insulin-like effect upon the glucose tolerance of normal and diabetic animals (2, 3) and the eviscerate animal (4). Does the stimulation of muscle suppress the rise in plasma amino acids following evisceration just as it suppresses the hyperglycemia of an eviscerate animal given a high glucose load? Under the conditions of the following studies there is a clear dissociation of the effects of insulin and of muscle work upon the levels of blood glucose and of plasma amino acids.

Male rats of the Sprague-Dawley strain were fed Archer Dog Pellets. At a weight of 250 ± 2 gm non-fasted rats were anesthetized by cyclopal sodium and functionally eviscerated. The stimulation of muscle to contract 5 times per second was by the procedure of Ingle (5). The left hind leg was weighted with 100 gm. In some experiments the stimulus passed from the biceps of the thigh to the foot of the left hind leg. In other experiments the stimulus passed from the right hind foot to the left hind foot, thereby activating all of the musculature of both hind legs. A solution of glucose, with and without insulin, was infused into the jugular vein by means of a continuous injection apparatus which delivered fluid from each of 6 syringes at the rate of 20 cc in 24 hours. The experiments lasted for 3–5 hours, whereupon the blood was collected by cannula from the abdominal aorta. Blood glucose was determined by the method of Miller and Van Slyke (6) and plasma amino acids by the method of Hamilton and Van Slyke (7). The conditions of each experiment are indicated in the tables.

Experiment 1 (table 1) involved resting and working rats without insulin. Muscle work increased glucose tolerance but failed to suppress the rise in plasma amino acids. There seemed to be a tendency, not reliably established by small samples, for muscle work to accelerate the rise in plasma amino acids.

Experiment 2 (table 2) shows that insulin suppresses the rise in plasma amino acids as well as the rise in blood glucose. Muscle work had an even more striking effect upon the level of blood glucose but failed to suppress the rise in plasma amino acids.

Goldstein *et al.* (8) have shown that both insulin and muscle work increase the transfer of glucose and other sugars across cell barriers. In the case of insulin, it has been suggested (9) that the increase in the rate at which glucose enters the cell represents the primary action of insulin to which all other consequences of insulin action are secondary. Since muscle work simulates the action of insulin upon glucose utilization and glucose space, its failure to have an insulin-like action upon the rise of amino acids following evisceration in the rat suggests that further analysis of these metabolic interrelationships is in order.

The new data reported in this discussion were obtained with the assistance of Gloria Torralba and Violeta Flores.

REFERENCES

1. INGLE, D. J. *Acta Endocrinol.* 17: 172, 1954.
2. INGLE, D. J., J. E. NEZAMIS AND K. L. RICE. *Endocrinology* 46: 505, 1950.
3. INGLE, D. J., J. E. NEZAMIS AND E. H. MORLEY. *Am. J. Physiol.* 165: 469, 1951.
4. INGLE, D. J. AND J. E. NEZAMIS. *Am. J. Physiol.* 155: 15, 1948.
5. INGLE, D. J. *Endocrinology* 34: 191, 1944.
6. MILLER, B. F. AND D. D. VAN SLYKE. *J. Biol. Chem.* 114: 583, 1936.
7. HAMILTON, P. B. AND D. D. VAN SLYKE. *J. Biol. Chem.* 150: 231, 1943.
8. GOLDSTEIN, M. S., V. MULLICK, B. HUDDLESTUN AND R. LEVINE. *Am. J. Physiol.* 173: 212, 1953.
9. LEVINE, R. AND M. S. GOLDSTEIN. *Rec. Progr. in Hormone Research.* In press.

INTERRELATIONS IN AMINO ACID AND CARBOHYDRATE METABOLISM

STUDIES OF THE NITROGEN SPARING ACTION OF CARBOHYDRATE WITH THE ISOLATED PERFUSED RAT LIVER[1]

LEON L. MILLER, WILLIAM T. BURKE[2] AND DAVID E. HAFT[2]

From the Departments of Radiation Biology and Biochemistry, School of Medicine and Dentistry, University of Rochester, Rochester, New York

SINCE the classical demonstration by Schroeder (1) that the liver was the site of urea formation in the dog, little has been done to evaluate quantitatively the contribution of the liver proteins and the blood proteins to endogenous urea formation. The nitrogen sparing action of carbohydrate in conserving protein nitrogen of endogenous origin as well as that of dietary origin has been extensively reviewed by Munro (2) who has succinctly summarized our knowledge of the mechanism with these words: "The nature of the mechanism by which carbohydrate spares protein is not known."

The recognized prominent role of the liver in intermediary protein and amino acid metabolism and the ease with which the isolated perfused rat liver may be maintained *in vitro* under essentially physiological conditions (3) render it uniquely suitable for a direct study of interrelations in nitrogen and carbohydrate metabolism.

This report describes liver perfusion experiments which for the first time permit a quantitative appraisal of the contribution of the liver and whole blood to endogenously produced urea and the nitrogen sparing effects thereon of glucose and fructose supplements. In addition, the overall nitrogen metabolism of an added complete mixture of 18 amino acids has been studied both in the absence and presence of glucose and fructose supplements. Further, it will be seen that the marked nitrogen sparing action of glucose and fructose could not in general be simulated by additions of corresponding large amounts of lactate, pyruvate, or α-ketoglutarate.

METHODS

Adult male rats of Wistar strain weighing generally from 200 to 350 gm were maintained on a diet of commercial rat food (Purina Checkers) until 18–20 hours before the start of the perfusion experiments when all food was withdrawn and the rats (both blood and liver donors) were allowed to have only drinking water until killed.

The preparation of the heparinized rat blood was carried out within the hour before the perfusion. The blood donor rats were lightly anesthetized with ether and exsanguinated by cardiac puncture with a syringe moistened with a heparin solution. Usually 12 ml of heparin (Liquaemin Roche diluted with Ringer's solution to 5 mg/ml) was used for about 130 ml of whole blood. The blood was then diluted with ⅓ volume of Ringer's solution and poured into the reservoir of the perfusion apparatus. Where solutions of carbohydrate were infused over the course of the experiment, the volume of diluting Ringer's was reduced by a corresponding amount. Details of the operation for the removal of the liver, after cannulation of the portal vein for inflow and the thoracic vena cava for outflow, and for its incorporation into the already prepared perfusion apparatus have been described previously (3).

The apparatus used here differed in a few minor respects from that used previously. Instead of a finger stall pump, the stripping action pump of DeBroske (4) was used. Further, the overflow return to the blood reservoir was connected with a 12-mm diameter Tygon tube directly to an outlet high on the blood reservoir side; this allowed oxygen to be passed into the air space of the reser-

[1] These studies were performed under contract with the United States Atomic Energy Commission at the University of Rochester Atomic Energy Project, Rochester, New York and were supported in part by research grants from the Jane Coffin Childs Memorial Fund for Medical Research, and from the Medical Research and Development Board Office of the Surgeon General Department of the Army under Contract No. DA-49-007-MD-451.

[2] Public Health Service Postdoctorate Research Fellow.

voir up through the overflow, into the 'lung' from which it was drawn with 'expired' carbon dioxide through the sintered glass bubble trap containing 40% potassium hydroxide. Lastly, in all of the perfusions in which more than 0.5 gm of carbohydrate were used, the material (glucose, fructose, lactate, pyruvate, or α-ketoglutarate) in excess of this amount was dissolved in distilled water (see below) and the solution infused at constant rate continuously over the entire course of the experiment through a fine bore polyethylene tube which was threaded down through the 'lung' to empty into the blood just before it passed into the pre-liver filter. The volume of fluid infused was kept constant (regardless of the amount of extra carbohydrate) and for the 4-hour duration of most of the experiments totaled 13.0 ml.

In some of the perfusions neither carbohydrate nor amino acid mixture was added; in others only carbohydrate supplements were added. Including the 0.5 gm of glucose or fructose added directly to the blood in the reservoir, the total dose of glucose or fructose was 0.5 gm, 1.2 gm, or 1.9 gm as indicated in the individual experiments.

In the perfusions in which lactate, pyruvate, or α-ketoglutarate were added, 0.5 gm of the sodium salt in neutral (pH 7.4) solution was added to the blood and the remainder infused continuously as noted above. In these experiments it was necessary to use 95% oxygen and 5% carbon dioxide instead of pure oxygen for oxygenating the blood in order to keep the blood pH down.

Wherever used, the dose of amino acids was the same and consisted of 160 mg of a mixture[3] of the essential amino acids and glycine plus 167 mg of a mixture[4] of the non-essential amino acids (totaling 41.6 mg of nitrogen of which 77.3% is α-amino acid nitrogen). The amino acids were neutralized (pH 7.2–7.4) with a small volume of 3.0 N NaOH and added directly to the blood before the onset of the perfusion. (In a number of the experiments L-lysine-6-C^{14}·HCl, or DL-lysine-6-C^{14}·HCl, or L-histidine-2-C^{14}·HCl in amounts of 5–12 mg, 6–12 microcuries of C^{14}, were included in the amino acid mixtures. These radioactivity studies will be reported elsewhere.)

Preparation of Liver and Plasma Labeled with L-lysine-6-C^{14}. For the first of the two experiments of table 2, three normal male rats were each injected intraperitoneally with DL-lysine-6-C^{14}·HCl (36 mg, 25 microcuries, in 6.5 ml of physiological saline). Twenty-four hours later the rats were exsanguinated with heparin, the blood pooled, the C^{14}-labeled plasma harvested and frozen until use 4 days later. At that time, 14.5 ml of the C^{14}-labeled plasma was added to the blood (to make a total volume of 155 ml) used to perfuse a normal rat liver for 9 hours. The expired C^{14}-labeled carbon dioxide was quantitatively collected in the potassium hydroxide trap described above. The total C^{14} activity found in the expired carbon dioxide corresponds to the fraction of the plasma protein which was oxidatively catabolized and was computed after totaling the C^{14} activity of the blood, the liver, and the expired carbon dioxide.

In the second experiment of table 2, a male rat weighing 190 gm was fed the Purina diet mixed with 50 mg of DL-lysine-6-C^{14}·HCl (35 microcuries) on each of two successive days. Forty-eight hours later the rat's liver was perfused by the usual procedure for 6 hours. Here the total C^{14} activity found in the 'expired' carbon dioxide corresponds to the fraction of the total C^{14}-labeled liver protein which was oxidatively catabolized in the 6 hours of the perfusion. In both experiments the C^{14} activity of all samples was measured by conversion to gaseous carbon dioxide (5) which was assayed in an ionization chamber coupled with a dynamic vane electrometer (6).

Chemical Methods. Serial samples of blood were withdrawn from the perfusion reservoir after thorough mixing at 0, ¼, ½, 1, 2, 3, 4 hours and analyzed in duplicate for glucose (7), fructose (8), α-amino acid nitrogen (9), urea nitrogen (10), lactate (11), and pyruvate or α-ketoglutarate (12). The analyses for urea nitrogen were carried out directly on aliquots of the blood. All of the other analyses were done on aliquots of an immediately prepared protein free filtrate (13). At the close of

TABLE 1. ENDOGENOUS UREA PRODUCTION BY ISOLATED PERFUSED LIVER BEST EXPRESSED IN TERMS OF BODY SURFACE AREA OF LIVER DONOR

Exp. No.	Liver Donor SA* cm^2	Urea-N Produced in 4 hr. *mg*	Urea-N/300 gm BW *mg*	Urea-N/300 cm^2 SA *mg*	Urea-N/10 gm Liver Wt. *mg*
153	420	16.7	14.3	11.9	11.3
154	456	19.7	14.9	12.9	19.7
155	425	18.4	15.6	13.0	16.7
156	423	14.8	12.6	10.5	14.5
166	370	13.2	13.9	10.7	17.6

* Surface area computed with the equation SA (in cm^2) = 0.78 (wt. in gm) + 148, based on data of Lee (16) for rats in the weight range used.

[3] L-Arginine HCl 800 mg, L-histidine HCl 400 mg, L-isoleucine 780 mg, L-leucine 1750 mg, L-lysine HCl 1230 mg, L-methionine 550 mg, L-phenylalanine 870 mg, L-threonine 740 mg, L-tryptophane 180 mg, L-valine 610 mg, and glycine 1000 mg.

[4] L-Glutamic acid 1024 mg, L-proline 320 mg, L-hydroxyproline 128 mg, DL-alanine 480 mg, DL-serine 488 mg, L-cysteine-HCl 112 mg, L-tyrosine 272 mg, and DL-aspartic acid 520 mg.

TABLE 2. OXIDATIVE CATABOLISM OF C^{14}-LABELED LIVER AND PLASMA PROTEINS: MEASURE OF THEIR CONTRIBUTION TO ENDOGENOUS UREA PRODUCTION

	*Exp. 1**. Plasma Proteins C^{14}-labeled (4.0 gm) *9 hr*	*Exp. 2**. Liver Proteins C^{14}-labeled (1.78 gm) *6 hr*
% Total C^{14} Activity at Start of Perfusion		
Liver Prot.	0	100
Plasma Prot.	100	0
% Total C^{14} Activity at End of Perfusion		
Liver Prot.	2.7	90.0
Plasma Prot.	95.5	6.3
C^{14} Activity in Total Expired CO_2 as % of Total Initial C^{14} Activity	1.5	3.7
Calculated C^{14}-labeled Protein N Catabolized/24 hr.	(10)(1.5)(4.0)(.16)(24/9) = 25.6 mg	(10)(3.7)(1.78)(.16)(24/6) = 42.1 mg

* These perfusions were carried out in collaboration with Dr. Chauncy G. Bly, Postdoctorate U. S. Atomic Energy Project Research Fellow, in 1952.

the perfusion weighed portions of the liver were in many instances analyzed for glycogen by appropriate methods (14). The rest of the liver was immediately homogenized in the cold with about five volumes of ice-cold distilled water, and the protein free filtrate prepared and analyzed in the same way as for the blood. In addition, determinations of the protein content of plasma and liver were carried out by micro-Kjeldahl or by the use of the modified Folin-Ciocalteau reagent (15).

An aliquot of the blood taken at zero time was incubated at the temperature of the perfusion (38–39.5°C) for the duration of the perfusion and glucose and lactate analyses were done in order to correct for changes related to glycolysis in the blood.

From the analytical data and the known volumes of blood removed in each sample as well as the volume of blood remaining at the close of the perfusions, the total urea nitrogen produced by the liver, the total α-amino acid nitrogen removed from the blood, as well as the glucose, fructose, pyruvate, lactate, and α-ketoglutarate balances have been computed.

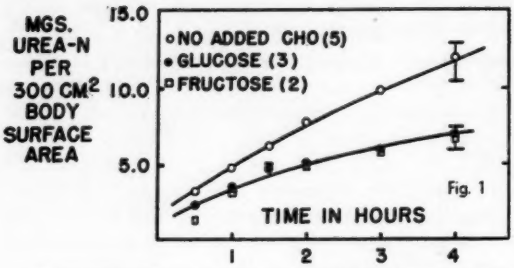
EXPERIMENTAL RESULTS

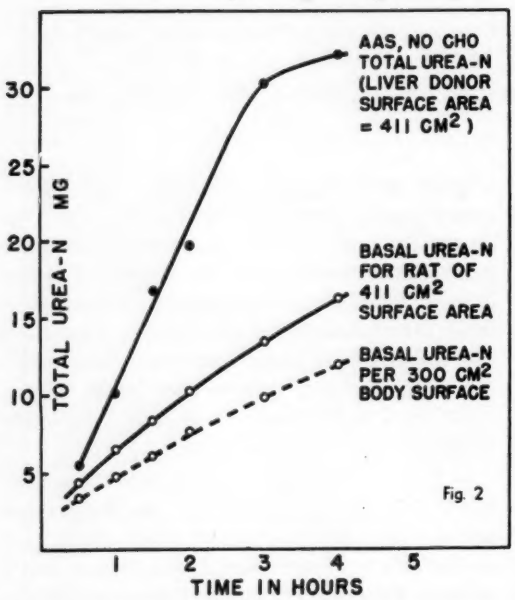
It will be noted that the urea production in the various experiments is expressed in terms of milligrams of urea nitrogen produced per 300 centimeter squared body surface area (of the liver donor). This mode of expressing the results was chosen because liver donor body surface area showed the best gross correlation with urea nitrogen production. In computing the surface area of the liver donors, we have used the data of Lee (16) to obtain the relation: surface area (cm^2) = (0.78) (body weight in gm) + 148, which gives a straight line valid for rats weighing 200–350 grams. Table 1 reveals that the urea nitrogen production per 300 grams body weight gives almost as good gross correlation. Obviously expressing urea production in terms of liver weight gives much poorer correlation. This apparently is not related to significant differences in total liver protein content because expressing urea production in terms of milligrams urea nitrogen per gram liver protein (not shown in the table) gave much poorer correlation than when expressed on a wet weight basis.

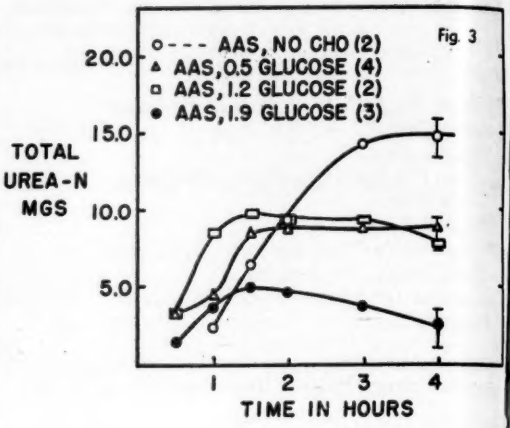
In figure 1 the upper curve depicts the endogenous urea nitrogen production by the isolated perfused liver under conditions which are perhaps comparable to those existing in the 18-hour fasted animal. Since no exogenous amino acids are added to the system, the urea produced must be primarily derived from deamination of free amino acids of the blood or liver. The fact that the free blood amino acid level (fig. 5) rises slowly during the course of the experiments implies that the perfused liver must be liberating not only enough free amino acids to supply the latter but also enough to yield an almost linear production of urea over the 4 hours of these experiments.

Although not shown in figure 1, in two experiments the addition of 0.5 grams of glucose to the perfusing blood produced no significant decrease in the endogenous urea production. However, the addition of 1.9 grams of glucose or fructose (*lower curve*, fig. 1) was associated with a remarkable sparing of nitrogen. The nitrogen sparing action is more pronounced after 2 hours and suggests that the nitrogen sparing action of carbohydrate requires this time interval before a maximal effect is elicitable.

It is of some interest to compute from our 4-hour liver perfusion data the mean endogenous







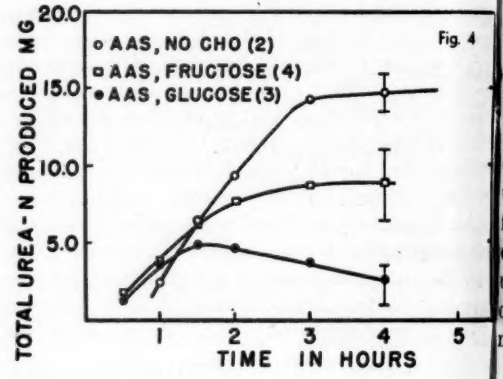


FIG. 1. Endogenous urea production by isolated perfused rat liver.

The curves in this figure and in figures 3 through 8 mean values obtained for the number of experiments indicated in parentheses. Wherever given, as in this figure, the I lines through the last points indicate the extremes of the range of values.

FIG. 2. Method of estimating urea production in excess of endogenous urea.

FIG. 3. Excess urea production by isolated perfused rat liver from a complete amino acid mixture. Added glucose spares nitrogen.

FIG. 4. Excess urea production by isolated perfused liver from a complete amino acid mixture. Added fructose spares nitrogen.

urea nitrogen production on a 24-hour basis per square meter of body surface area for comparison with known data for the 24-hour nitrogen output in the rat (17) on a non-protein diet.

$(11.9)(6)(1 \times 10^4/3 \times 10^2) = 2.4$ gm urea N/m² (no carbohydrate supplement)

$(7.0)(6)(1 \times 10^4/3 \times 10^2) = 1.4$ gm urea N/m² (1.9 gm fructose or glucose supplement)

This is to be compared with 1.91 grams of nitrogen per square meter estimated by Bricker and Mitchell (17) for rats on a non-protein intake.

The data of table 2 clearly point to the fact that both the liver and circulating plasma proteins contribute to the endogenous urea production. In *experiment 1* of table 2 we see that 1.5% of the C^{14}-label of lysine-labeled plasma protein was oxidized in the course of 9 hours of perfusion. Since 100 milliliters of plasma with a protein content of 4.0% was used in the first experiment on a 24-hour basis one might expect the liver to produce 25.6 milligrams of urea nitrogen.

The donor of this liver weighed 220 grams, therefore had a surface area = .78 W + 14 = 320 cm². On the basis of the observed endogenous production the perfused liver of a rat with a 320 centimeter squared surface area would yield 6 (11.9) (320/300) = 76.5 mg urea N/24 h. Thus catabolism of plasma protein could yield (25.6/76.5) 100 = 33% of the urea nitrogen.

Similarly, from *experiment 2* of table 2 we may estimate that 42.1 milligrams of urea nitrogen

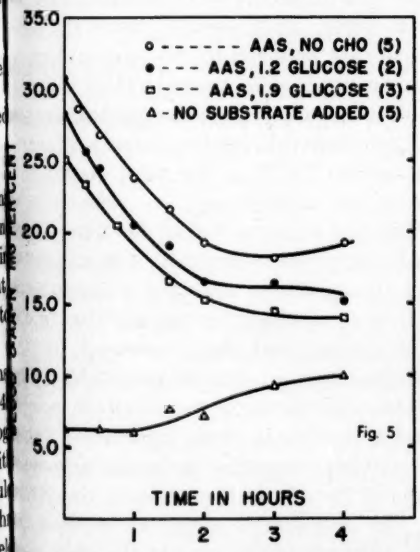
derivable from liver protein in 24 hours. The liver donor here had a surface area of 296 centimeter squared; on this basis a 24-hour estimated production of 72.5 milligrams of urea nitrogen can be calculated. Thus the liver protein here yields 58% of the endogenous urea produced. The small deficit of about 9% is easily derivable from the catabolism of hemoglobin released by normal red cell breakdown.

Figure 2 depicts the essential data involved in computing the urea nitrogen produced in excess of the basal endogenous urea in experiments in which the amino acid mixture was added with or without glucose or fructose supplements. First, the surface area in centimeter squared of the liver donor was computed, then from the data of figure 1 the anticipated basal endogenous urea production was estimated for a liver fron an animal of the computed surface area, as shown in the middle curve of figure 2. The values obtained by subtracting the estimated basal urea production from the observed total urea production correspond to the urea production referable to the amino acid supplement. Thus all subsequent figures present curves which represent the differences between the basal urea production and the urea production observed under the various conditions of amino acid and carbohydrate supplementation.

In figure 3 are recorded the effects on urea production incident to the addition of the amino acid mixture with and without graded amounts of glucose. In contrast to the lack of effect on the basal urea production the addition of 0.5 grams of glucose along with the amino acid mixture results in a significant nitrogen sparing effect particularly after the first 2 hours of the experiments. Increasing the amount of glucose added to 1.2 grams did not significantly enhance this effect. However, increasing the total glucose added to 1.9 grams did result in a further increase in nitrogen sparing as measured by the depression of urea production but not as measured in terms of nitrogen balance, as is apparent from table 4.

Figure 4 affords a comparison between the nitrogen sparing effect of equal (1.9 gm) glucose and fructose supplements, and reveals that, although the two sugars in large doses were equally effective in suppressing the basal endogenous urea production, glucose is definitely superior to fructose in sparing nitrogen when the amino acid supplement is added. In all the experiments of figures 3 and 4 the nitrogen sparing effect assumes significant magnitude only after 1½–2 hours.

Figures 5 and 6 contain average curves of the blood free α-amino acid concentration changes and of the cumulative disappearance of the α-amino nitrogen respectively in the experimental groups of figure 3. They reveal that the increasing nitrogen sparing effect of the (0.5 and 1.2-gm glucose) carbohydrate cannot be interpreted entirely on the basis of altered clearance of α-amino nitrogen from the blood. Similarly,



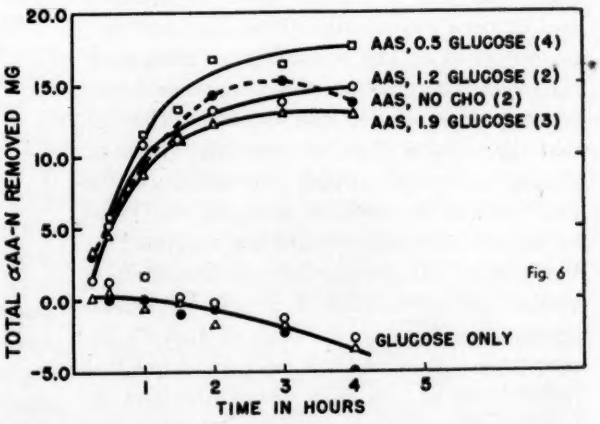


FIG. 5. Changes in blood amino acid concentration during isolated rat liver perfusion.
FIG. 6. Cumulative disappearance of blood amino acid nitrogen during isolated liver perfusion with amino acids.
See legend to figure 1 for explanation of curves.

TABLE 3. CHANGES IN RATIO LIVER FREE AA N CONC./BLOOD FREE AA N CONC. RELATED TO ADDITION OF GLUCOSE, FRUCTOSE, PYRUVATE, α-KETOGLUTARATE AND LACTATE IN THE ISOLATED PERFUSED RAT LIVER

	(1) Mean Final Blood Free α-AA N Conc. mg %	(2) Mean Final Liver Free α-AA N Conc.* mg %	Ratio (2)/(1)
No Substrate (4)	8.8 (4.7–17.0)	72.0 (44.0–127.)	8.2
AAs, No CHO(2)	20.2 (19.8, 21.6)	160. (124.0, 195.0)	7.9
1.9 Gl(2)	9.0 (8.0, 9.9)	43.3 (40.4, 46.5)	4.8
AAs + 0.5 Gl(4)	16.9 (16.3–17.2)	66.2 (43.8, 75.6)	3.9
AAs + 1.2 Gl(2)	16.7 (13.7–18.1)	60.2 (57.2–66.7)	3.6
AAs + 1.9 Gl(3)	14.2 (12.4–15.7)	44.0 (40.6–45.9)	3.1
AAs + 1.9 Fr(4)	20.4 (18.9–21.4)	65.7 (56.0–78.2)	3.2
AAs + Pyruvate(3)	19.6 (15.4–21.7)	41.5 (26.7–40.1)	2.1
AAs + α-Ketoglutarate(3)	21.9 (17.1–27.1)	46.2 (28.1–58.5)	2.1
AAs + Lactate(3)	17.4 (16.4–18.1)	49.0 (32.3–77.5)	2.8

* These values are uncorrected for the extracellular free amino acids and are therefore probably lower than the true intracellular free amino acid levels.

the bottommost curve of figure 6 indicates that the nitrogen sparing effect of the large dose of glucose in the absence of added amino acids is not reflected in the blood free α-amino acid. Nor in the experiments without the amino acid supplement is the total quantity of free α-amino acid nitrogen released into the circulating blood significantly altered by increasing the amount of glucose added. In this connection table 3 reveals that the addition of glucose or fructose profoundly affects the ratio of the final liver free α-amino acid nitrogen concentration to the final (at 4 hr.) concentration of free α-amino acid nitrogen in the blood. The addition of the amino acid supplement alone did not alter the ratio. The fact that the addition of glucose alone almost halved the free amino acid nitrogen concentration in the liver without significantly changing the blood amino acid nitrogen concentration suggests that the carbohydrate has depressed the breakdown of protein. An acceleration of protein resynthesis appears less likely on the basis of Tarver's observations (18) in which it has been found that maintenance of rats on a low protein diet increases liver plasma protein turnover time markedly over that found in rats on an isocaloric high protein intake.

The data for lactate, pyruvate and α-ketoglutarate in tables 3 and 4 clearly indicate that a lowered liver amino acid to blood amino acid concentration ratio does not per se favor positive nitrogen balance, since with the exception of the single (2.5 gm) pyruvate experiment the nitrogen 'balance' is almost as badly negative here as with no carbohydrate supplement whatsoever. Here again, however, as also in the case of the large fructose supplements, conditions yielding a badly negative nitrogen 'balance' in the isolated liver system might be considerably more favorable in an intact animal where amino acids, not taken up and catabolized by the liver, become available to the non-hepatic tissues for net protein synthesis. This latter conjecture may conceivably be the basis for the strongly enhanced positive nitrogen balance in growing animals given a surfeit of calories (2).

Table 4 supports these conclusions and presents the results of subtracting the net average excess production of urea from the total amino acid nitrogen which has disappeared in the course of the 4-hour perfusion. The differences termed net ΔN 'balance' reveal that the glucose supplements were much more effective than the fructose supplements. These values of net ΔN 'balances' would undoubtedly be low (unless corrected as they are) insofar as the total amino acid nitrogen removed is significantly greater than the estimated α-amino nitrogen because the ninhydrin procedure used does not measure the non-α amino nitrogen present in arginine, histidine, lysine, proline, oxyproline or tryptophane. In the correction made, it is assumed that all these amino acids are removed to the same extent as the α-amino nitrogen in general. Thus the corrected nitrogen 'balances' are considerably larger, since the non-ninhydrin reactive amino acid nitrogen amounts to 22.7% of the total amino acid nitrogen in the mixture used.

Although the range of values for total blood α-amino nitrogen removed in table 4 points to the possibility that the total clearance of amino acids by the liver is enhanced by the addition of 0.5 grams of glucose, and then decreased by the larger supplements of glucose or fructose, the number of experiments is too small to permit statistical evaluation of these differences. Yet it suggests another component of the nitrogen sparing action of carbohydrate as seen in the intact animal, viz. diversion of amino acids from the catabolic influence of the liver to the peripheral tissues where the amino acids may be available for protein synthetic needs.

Inability of Pyruvate, Lactate, or α-Keto

TABLE 4. EFFECT OF GLUCOSE, FRUCTOSE, PYRUVATE, α-KETOGLUTARATE AND LACTATE ON UREA PRODUCTION, BLOOD AA N REMOVED, N BALANCE AND LIVER GLYCOGEN SYNTHESIS IN ISOLATED PERFUSED LIVER SYSTEM*

(1) Av. SA *cm*	(2) Supplement to AAs in gm† (No. of Expts.)	(3) 4 hr Total Urea N Produced *mg*	(4) 4 hr Total Urea N in Excess of Basal *mg*	(5) Total Blood α-Amino Acid N Removed *mg*	(6) Total Blood Amino Acid N Removed *mg*	(7) Net N Balance (6)–(4) *mg*	(8) N Balance (6)–(3) *mg*	Final Liver Glycogen Content *gms % wet wt.*
410	None (2)	30.9	14.6	13.2 (12.4, 14.4)	17.1	+2.5	−13.8	
377	Gl 0.5(4)	23.9	8.9	17.7 (14.1–21.2)	22.9	+14.0	−1.0	(0.17, 0.40)‡
370	Gl 1.2(2)	22.2	7.6	14.9 (13.9, 15.7)	19.3	+11.7	−2.9	0.69, 2.47
391	Gl 1.9(3)	18.1	2.5	12.8 (10.0–16.0)	16.6	+14.1	−1.5	0.51–1.25
343	Fr 1.2(1)	18.5	4.9	7.4	9.6	+4.7	−8.9	
410	Fr 1.9(4)	24.4	8.7	11.6 (8.0–16.9)	15.1	+6.4	−8.3	2.6, 3.9
372	Pyr 1.9(2)	28.1	13.2	12.5 (16.1, 8.8)	16.1	+2.9	−12.0	.03–.12
362	Pyr 2.5(1)	12.8	−1.8	6.6	8.5	+10.3	−2.5	.12
340	α-Ketoglutarate 1.0(3)	21.2	7.7	8.1 (4.6–12.1)	10.5	+2.8	−10.7	0.03–0.14
388	Lactate 1.0(3)	30.7	15.0	12.9 (12.3–13.4)	16.5	+1.6	−14.1	0.26–0.70

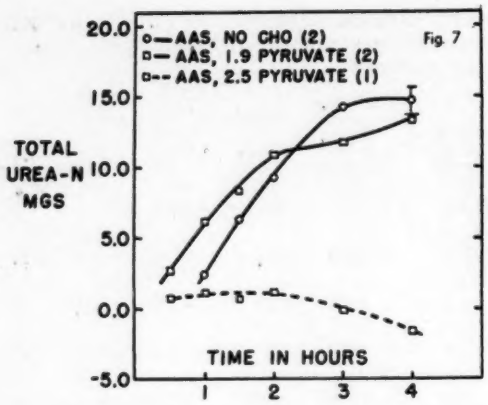
* Values given in this table are averages with the separate values or ranges indicated in parentheses.
† Number of experiments in parentheses.
‡ These final glycogen values were obtained from livers of two normal 18-hr. fasted rats other than those to which the rest of the data pertains. They were perfused with 0.50 gm glucose but no amino acids.

glutarate to Spare Nitrogen in the Isolated Perfused Liver. In only one of three experiments (table 4) did the addition of 2.5 grams of sodium pyruvate to the amino acid mixture result in nitrogen sparing quantitatively similar to that associated with a comparable amount of glucose. Qualitatively, the effect of the large dose of pyruvate in depressing the clearance of amino acids was even more noteworthy. The average curve for two experiments with 1.9 grams of sodium pyruvate, however, does not differ from that obtained with no added carbohydrate (fig. 7). Similarly, figure 8 reveals the relative inefficiency of lactate in three experiments in which 1.0 gram of sodium *d*-lactate was introduced with the amino acid mixture. The urea production of livers perfused with amino acids and added α-ketoglutarate are not presented in detail here. However, table 4 reveals the apparent nitrogen sparing effect in the urea nitrogen produced in excess of the endogenous urea. This effect is obviously primarily related to the greatly diminished uptake of amino acids by the liver commented on previously, and the nitrogen sparing as manifest in the nitrogen 'balance' is probably insignificantly small.

Glucogenesis and Glycogenesis from Fructose, Lactate, Pyruvate and α-Ketoglutarate. The data obtained on the metabolic conversion of pyruvate or α-ketoglutarate to glucose are not presented here in detail. However, they indicate that the rate of conversion of these substances to glucose is not nearly as rapid nor as extensive as the conversion of fructose to glucose. This may, at least in part, explain the non-uniformity and relative inefficiency of these substances in sparing nitrogen under these conditions.

In the experiments with large doses (1.9 gm) of fructose, 80–90% of the fructose was cleared from the blood; of this, about 40% was accounted for as blood glucose (non-fructose reducing sugar), and about 15% as liver glycogen. The fructose unaccounted for was not converted to lactate or pyruvate. The amounts of the latter found by analysis were much too small to account for more than 10% of the deficit. Lundsgaard (19) has described cat liver perfusions of 1½ or 2 hours duration with relatively much smaller doses of fructose which were in the main converted to blood glucose and liver glycogen. He was unable to obtain significant glycogenesis from glucose perfused through the cat liver.

In the lactate perfusions, 40–50% disappeared from the blood. About ⅔ of this was accounted for as blood glucose. In the pyruvate experiments about 75% of the pyruvate disappeared from the blood, with ⅓ of this amount accounted for as blood glucose. In both the pyruvate and lac-



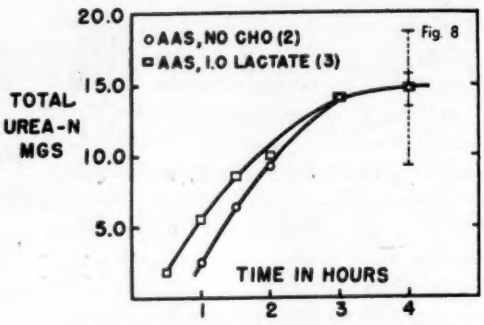


FIG. 7. Excess urea production by isolated perfused rat liver from a complete amino acid mixture with pyruvate.

FIG. 8. Excess urea production by isolated perfused rat liver from a complete amino acid mixture with lactate.

See legend to figure 1 for explanation of curves.

tate experiments, small amounts of liver glycogen, not exceeding 0.2 gm %, were present (table 4).

In the α-ketoglutarate experiments about 80% of the ketoglutarate disappeared from the blood, with less than ⅙ of this accounted for as blood glucose, and a maximum of 0.14 gm % of liver glycogen.

The relatively massive liver glycogen synthesis from the large doses of glucose or fructose (table 4) invites comment for several reasons. Firstly, the ability of the isolated liver to synthesize and retain glycogen in amounts as high as 3.9 gms %, must be accepted as an additional property in witness of the viability and physiological integrity of the isolated perfused rat liver over periods of at least 4–6 hours. Only by raising the potassium ion concentration in the medium to abnormally high levels were Hastings and his collaborators able to effect glycogenesis of this magnitude in liver slice systems (20). Secondly, the liver glycogen data of table 4 reveal no readily apparent correlation with the extent of nitrogen sparing as measured by nitrogen 'balance.'

DISCUSSION

The use of the isolated perfused rat liver in an attempt to arrive at a better understanding of the mechanism of the nitrogen sparing action of carbohydrate has yielded not only information bearing directly on that problem but also having implications for the basic problem of designing an *in vitro* system for studying net protein synthesis in mammalian tissue. Furthermore, the results obtained point to the liver and blood proteins as being the major source of endogenous urea nitrogen. In addition, they emphasize the contribution of the liver and blood proteins toward maintainance of the blood free amino acid level particularly under conditions where no exogenous amino acids are forthcoming.

Any hypothesis for explaining the mechanism of the nitrogen sparing action of carbohydrate must explain this action as it occurs both in the absence and in the presence of exogenous amino acids. Such an hypothesis should be applicable to the isolated perfused liver, since the data clearly demonstrate that large amounts of glucose or fructose (which an intact rat might assimilate in 4–6 hours while consuming a non-protein diet adequate in calories), exert a pronounced nitrogen sparing effect manifest in depressed production of urea, whether exogenous amino acids are added or not.

In the present state of our knowledge, oxidative deamination of amino acids is in large measure best viewed as being channeled through the L-glutamic acid dehydrogenase system in concerted action with a number of α-ketoglutaric-amino acid transaminases (21). Figure 9 schematically visualizes the central role which liver L-glutamic acid dehydrogenase and its essential coenzymes DPN (or TPN) may play in controlling the rate of ammonia and thereby the rate of urea formation. The work of Krebs has shown that in the normal liver the rate of urea formation may be regarded as a function of the ammonia concentration. The two major ammonia forming reactions in the liver are: *a*) oxidative deamination of amino acids as manifest primarily in the glutamic dehydrogenase system of figure 9; *b*) the

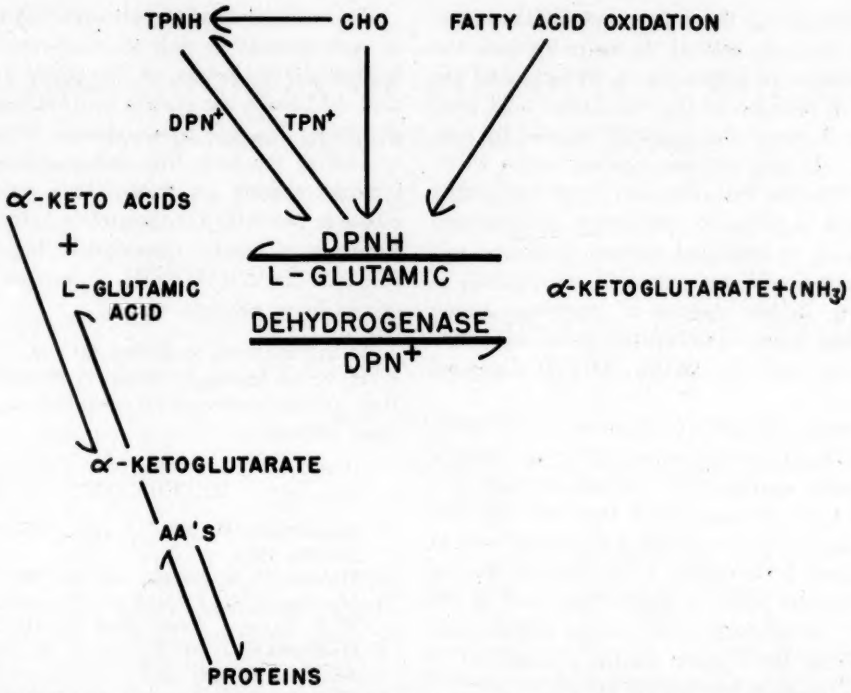


FIG. 9. Relations of liver L-glutamic acid dehydrogenase system to *a*) Coenzyme I in reduced (DPNH) and oxidized (DPN^+) form; *b*) free liver amino acids (AA's); *c*) fatty acid oxidation and *d*) carbohydrate oxidation, which in the case of glucose may also yield reduced Coenzyme II (TPNH) from oxidized Coenzyme II (TPN^+).

cleavage of the amide group of glutamine as mediated by the liver glutaminases–phosphate catalyzed (22) and pyruvate catalyzed (22)–or by the glutamine transaminase system of Meister (23).

From the glutamic dehydrogenase catalyzed reaction of figure 9 we may derive the following mass law expression:

$$[NH_3] = K\left(\frac{[DPN^+]}{[DPNH]} \cdot \frac{[\text{Glutamate}]}{[\text{Ketoglutarate}]}\right)$$

Other factors being equal, the $[NH_3]$ may then be regarded as a function of the two ratios $[DPN^+]/[DPNH]$ and [Glutamate]/[Ketoglutarate]. Of these two ratios it is of interest to consider the effect of small changes of $(DPN^+]$ on the numerical value of the former ratio assuming the total concentration of both forms of the coenzyme to remain constant and the ratio to have an initial value of unity. For example, a 10% increase in $[DPN^+]$ with a corresponding decrease in [DPNH] would enhance the $[NH_3]$ by a factor of 1.22; a 20% increase in $[DPN^+]$ similarly would enhance the $[NH_3]$ by a factor of 1.50; a 30% increase in $[DPN^+]$ would enhance the $[NH_3]$ by a factor of 1.85; and a 40% increase would more than double the $[NH_3]$ with a factor of 2.33. Conversely, metabolic processes which build up [DPNH] at the expense of $[DPN^+]$ would indirectly depress the formation of ammonia by the glutamic dehydrogenase system. Figure 9 reveals also how the [DPNH] may tend to build up especially from glucose metabolism, not only via the conventional glycolytic pathway but also via the Warburg-Dickens hexose monophosphate shunt pathway. This pathway has been found to be quantitatively of major importance in the liver (24). Figure 9 also indicates that the oxidative metabolism of fatty acids may significantly enhance the [DPNH]. Here it is pertinent to mention that dietary fat can exert a pronounced nitrogen sparing action (2).

The factors which may directly or indirectly affect the ratio [Glutamate]/[Ketoglutarate] should also be considered. In the absence of specific data on the effects of potential nitrogen sparing substances upon the intracellular concentration of these metabolites, extended speculation is not warranted. It must be emphasized that this ratio is probably not quantitatively as sensitive as the coenzyme ratio because the sum of the intracellular concentrations of glutamate and α-ketoglutarate is not as constant, nor are the

concentrations of these two metabolites necessarily reciprocally related. As figure 9 shows, the concentration of glutamate is directly and indirectly a function of the free amino acid level which is in every case (table 3) reduced by substances with any nitrogen sparing action whatsoever. Whether this reduction in the free amino acid level is primarily the result of depressed proteolysis, or mediated through unknown cellular amino acid concentrating mechanisms is unknown. In the absence of exogenous amino acids, the former mechanism seems in better agreement with the known facts as discussed above.

Presumably an effect on the level of free amino acids in the liver is per se not a definitive index of the nitrogen sparing action of carbohydrate. The data of table 3 clearly show that the final free amino acid level in the liver is decreased both in the glucose experiments, with nitrogen sparing almost to the point of equilibrium, and in the fructose, ketoglutarate and lactate experiments. In the latter the nitrogen sparing as measured by nitrogen 'balance' was modest at best.

The potential contribution of glutamine to the formation of liver ammonia and urea is unquestionably important in the intact animal where the blood glutamine transports peripherally formed ammonia to the liver. Without here opening into the controversial issue of the role of glutamine in urea formation by the liver, it seems proper to mention that glutamine nitrogen (in unpublished experiments) is converted to urea by the isolated perfused rat liver at a rate greatly exceeding that found for the complete amino acid mixture. It appears probable that the small but sharp rise in blood urea seen in most of the perfusion experiments in the first half hour (e.g. fig. 1) is largely referable to the rapid conversion of the donor blood glutamine nitrogen to urea.

SUMMARY

The isolated perfused rat liver has been used along with lysine-6-C^{14} to demonstrate that the major portion of endogenous urea nitrogen is derivable from catabolism of the blood and liver proteins.

On the basis of nitrogen 'balance' calculations, glucose is definitely superior to fructose in sparing the nitrogen of an added complete amino acid mixture. However, both sugars depress endogenous production to an equal extent. Supplements of lactate, pyruvate, and α-ketoglutarate are relatively inefficient in sparing the nitrogen of an added complete amino acid mixture.

The mechanism of the nitrogen sparing action of carbohydrate as well as other metabolites is interpreted in terms of the liver L-glutamic acid dehydrogenase system coupled with α-ketoglutaric-amino acid transaminases. Observed decreased in the liver free amino acid level with nitrogen sparing are related to a potential decrease in the ratio [Glutamate]/[α-ketoglutarate]. Probably of greater quantitative importance is the ratio [DPN^+]/[DPNH], a decrease in which should favor nitrogen sparing.

We are indebted to Helen daCruz, Gilda DeF. Wolk, Sarah Hallagan, Mary F. Phillips, Maxine Raz, and Nellie Socala for technical assistance in these studies.

REFERENCES

1. Schroeder, W. *Arch. f. exp. Path. u. Pharm.* 15: 365, 1881.
2. Munro, H. N. *Physiol. Rev.* 31: 449, 1951.
3. Miller, L. L., C. G. Bly, M. L. Watson and W. F. Bale. *J. Exper. Med.* 94: 431, 1951.
4. DeBroske, J. M. F. and L. R. Crisp. *Rev. Sci. Inst.* 23: 381, 1951.
5. Van Slyke, D. D., J. Plazin and J. R. Weisiger. *J. Biol. Chem.* 191: 299, 1951.
6. Bale, W. F. Unpublished observations.
7. Nelson, N. *J. Biol. Chem.* 153: 375, 1944.
8. Weichselbaum, T. E., H. W. Margraf and R. Elman. *Metabolism* 2: 434, 1953.
9. Moore, S. and W. H. Stein. *J. Biol. Chem.* 176: 367, 1948.
10. Conway, E. J. and E. O'Malley. *Biochem. J.* 36: 655, 1946.
11. Barker, S. B. and W. H. Summerson. *J. Biol. Chem.* 138: 537, 1941.
12. Friedemann, T. E. and G. E. Haugen. *J. Biol. Chem.* 147: 417, 1943.
13. Hagedorn, H. C. and B. N. Jensen. *Biochemisch. Ztschr.* 135: 46, 1923.
14. Good, C. A., H. Kramer and M. Somogyi. *J. Biol. Chem.* 100: 490, 1933.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. *J. Biol. Chem.* 193: 265, 1951.
16. Lee, M. O. *Am. J. Physiol.* 89: 24, 1929.
17. Bricker, M. L. and H. H. Mitchell. *J. Nutrition* 34: 491, 1947.
18. Steinbock, H. L. and H. Tarver. *J. Biol. Chem.* 209: 127, 1954.
19. Lundsgaard, E. *Bull. Johns Hopkins Hosp.* 63: 90, 1938.
20. Hastings, A. B., C. T. Teng, F. B. Nesbett and F. M. Sinex. *J. Biol. Chem.* 194: 69, 1952.
21. Cohen, P. P. In: *Chemical Pathways of Metabolism*, Vol. 2, edited by D. M. Greenberg New York: Academic Press, 1954, p. 43.
22. Errera, M. *J. Biol. Chem.* 178: 483, 1949.
23. Meister, A. *J. Biol. Chem.* 210: 17, 1954.
24. Bloom, B. and DeW. Stetten, Jr. *J. Biol. Chem.* 212: 555, 1955.

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Biochemistry of Disease[1]

Chairman: M. H. POWER

PORPHYRINS AND PORPHYRIN PRECURSORS IN HUMAN AND EXPERIMENTAL PORPHYRIA

SAMUEL SCHWARTZ

From the Department of Medicine, University of Minnesota Medical School, Minneapolis, Minnesota

THE past decade has witnessed a considerable acceleration of basic research in the field of porphyrin metabolism, and an increasing fusion of the interests of biochemists and clinicians as the role of the free porphyrins and their precursors in the biosynthesis of metalloporphyrins such as hemoglobin, cytochromes, catalase, chlorophyll, etc., becomes ever more clear. In keeping with the theme of this symposium, major attention will be devoted here to a consideration of porphyria and of the stimulus given by this relatively rare disease to our understanding of the broader aspects of porphyrin metabolism. In addition, some salient aspects of porphyrin chemistry and synthesis will be considered briefly, especially as they are necessary to an understanding of the subsequent discussion.

PORPHYRIN CHEMISTRY AND BIOSYNTHESIS

The elegant studies of Shemin and his collaborators in this country and of Neuberger and coworkers in England have shown that the carbon atoms of porphyrin are derived from the two carbons of acetate and from the methylene carbon of glycine, while the four nitrogen atoms are derived from glycine (1). As shown in figure 1, these investigators defined certain early derivatives with eventual formation, via a succinyl CoA intermediate, of delta-amino levulinic acid. Two molecules of the latter compound were shown to condense to form porphobilinogen, which appears to be the common obligatory precursor of the various porphyrins.

In addition to protoporphyrin shown in figure 1, we shall be concerned here primarily with the biological significance of uroporphyrin and coproporphyrin. The former is an octocarboxyl porphyrin, having four acetic acid and four propionic acid side chains, while the latter is a tetracarboxyl porphyrin in which the four acetic acid groups of uroporphyrin have been replaced by methyl groups.

On heating in dilute acid, porphobilinogen is readily converted to uroporphyrin (2). Incubation with chicken blood (3), chlorella (4), or rat liver homogenate (5), however, leads to its enzymatic conversion to copro-, proto-, (both free and heme), and several other porphyrins. A considerable part of the porphyrin found in liver homogenate incubated with porphobilinogen is present as a non-fluorescent 'pro-porphyrin' which is readily converted to the fluorescent porphyrin by the addition of iodine or brief exposure to ultraviolet light. Some properties of these various compounds are summarized in table 1.

The question of heme synthesis from porphyrin and the possible interconversion of porphyrins is still unsettled. Until recently it was assumed that the uroporphyrin formed from porphobilinogen was progressively decarboxylated through coproporphyrin to protoporphyrin. The latter then was assumed to complex with iron to form heme. Various investigators have reported what they considered to be both direct and indirect evidence in accord with this concept.

Recent studies in our laboratory (6) have failed to confirm the progressive decarboxylation theory. Uroporphyrin C^{14} and coproporphyrin C^{14} having up to 190,000 counts per minute per milligram were incubated with liver homogenate

[1] San Francisco, April 13, 1955.

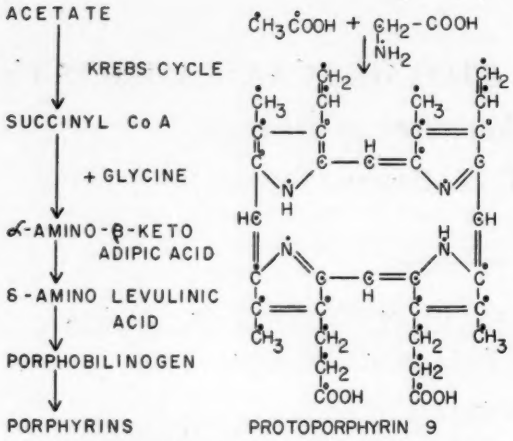


FIG. 1. Biosynthesis of porphyrin from acetate and glycine. (Corresponding C^{14} and N^{15} atoms are identified by ● or ○ labeling.)

and bone marrow, perfused through liver, or injected into rats and rabbits. Though decarboxylation of as little as 0.1 μgm, or less than 0.1% of the added dose, would have been readily detected, no activity was found in the purified coproporphyrin, protoporphyrin, or heme isolated in the uroporphyrin C^{14} studies, nor was any activity found in the protoporphyrin or heme in the studies employing coproporphyrin C^{14}. Significant activity was, however, found in the heme isolated from bone marrow or liver incubated with protoporphyrin C^{14}. Granick too has reported significant labeling of heme in chicken erythrocytes incubated with protoporphyrin (unlabeled) and radioactive iron (7). Unless the above studies are vitiated by the lack of essential cofactors in the systems employed, one must, therefore, conclude that uro-, copro-, and protoporphyrin are formed independently from porphobilinogen. Other possible green porphyrin derivatives of uro- and coproporphyrin will be discussed below.

SOME GENERAL COMMENTS ON PORPHYRINS IN TISSUES AND EXCRETA

It is quite likely that porphyrin synthesis takes place in all body tissues. Data on the content of free porphyrins is most abundant for blood, marrow, and liver. In these tissues approximately 90–95% of the total extractable porphyrin is protoporphyrin, most of the remainder being coproporphyrin.

Erythrocyte protoporphyrin concentration is increased in conditions which are or appear to be associated with iron deficiency, such as blood loss anemia, lead toxicity, anemia of infection and

TABLE 1. CHARACTERIZATION OF PORPHYRIN AND SOME OF ITS PRECURSORS

	Porphobilinogen	Pro-porphyrins[1]	Porphyrins
Color	0	?	Red-brown
Ehrlich reaction[2]	+	0	0
Red fluorescence[3]	0	0	+
Heat, pH 1–4 → porphyrin[4]	+	+	−
I_2, Brief UV → porphyrin	0	+	−
Demonstrable in normal urine[5]	0	+	+

[1] The identity of these compounds with the colorless porphyrinogens has not been established. Some, at least, appear to be colored.

[2] Red color appears on addition of Ehrlich's reagent (0.24% *p*-dimethyl amino-benzaldehyde in 7 N HCl. The color is intensified only slightly by the addition of an excess solution of saturated sodium acetate. The red aldehyde is not extracted by chloroform, in contrast to the red urobilinogen aldehyde formed similarly. [3]Best excited with 405 mμ light from a mercury lamp after acidification with HCl. [4]Mixture of uroporphyrin isomers (I–IV) formed from porphobilinogen. [5]Pro-coproporphyrin and coproporphyrin present in approximately equal amount in normal urine. Traces of uroporphyrin and 8 or more other porphyrins also present.

uremia. Erythrocyte coproporphyrin concentration is related primarily to the rate of erythropoiesis, values averaging approximately 1 μgm per 100 ml erythrocytes for each per cent reticulocytes (i.e., 5 μgm copro-/100 ml erythrocytes with 5% reticulocytes, etc; ref. 8, 8a).

Per diem urinary excretion of coproporphyrin averages approximately 180 μgm in normal adult males and 140 μgm in females (9). Aside from porphyria (see below) values are most elevated in individuals exposed to lead. Increased erythropoiesis, alcoholism, and liver dysfunction are generally associated with moderately elevated values. Renal disease with uremia leads to decreased porphyrin excretion.

Normal fecal coproporphyrin excretion ranges from approximately 400 to 1000 μgm per day. Fecal protoporphyrin is related both to bacterial breakdown of hemoglobin in the gastrointestinal tract and to endogenous factors which are not understood.

HUMAN PORPHYRIA

The major types of human porphyria and their distinguishing features are summarized in table

TABLE 2. DISTINGUISHING FEATURES OF HUMAN PORPHYRIA*

	Erythropoietic	Hepatic	
		Acute, Intermittent	Cutanea Tarda
Synonyms	Congenital or photosensitive	Acute toxic or idiopathic	Chronic
Frequency	±2–4%	±60–70%	±30–40%
Usual age of onset	Childhood	2d to 4th decade	4th and 5th decade
Clinical findings	Photosensitivity Splenomegaly and hemolytic anemia; Erythrodontia	Abdominal colic; Paresis and paralysis; Psychic disturbances; Hypertension; Oliguria; Constipation	Photosensitivity; Skin pigmentation; Hepatic dysfunction
Urine findings			
Color	Red	May be normal (darkens on standing)	Red
Porphobilinogen	Absent	Present	Absent
Zn porphyrin	Absent	Present	Present
Uroporphyrin	Type I	Types I and III	Types I and III
Marrow porphyrin	Markedly increased	Normal	Normal
Liver porphyrin	Slightly increased	Markedly increased (chiefly in precursor form)	Markedly increased (chiefly as porphyrin)

* Article by author, "Clinical Aspects of Porphyrin Metabolism", VA Tech. Bulletin, 10–94, 1953.

2. Chemically erythropoietic porphyria is distinguished primarily by the presence of increased amounts of porphyrin in the bone marrow, while in hepatic (acute intermittent and cutanea tarda) porphyria, excessive porphyrin concentration is seen chiefly in the liver (10). Large amounts of porphobilinogen are found in the liver and excreted in the urine only of patients with the acute intermittent disease. Clinically the latter group of patients show neurological and psychiatric disturbances as well as abdominal colic as their primary complaints, whereas the others exhibit chiefly manifestations of photosensitivity. Other pertinent features may be summarized as follows:

Erythropoietic Porphyria. Examination of unstained smears of bone marrow by fluorescence microscopy shows the porphyrin fluorescence to be localized chiefly to the nuclei of normoblasts. A second abnormality in many of these cells is the presence within the nucleus of a non-fluorescent area which is presumed to contain hemoglobin because it stains characteristically with benzidene and because it has intense light absorption at about 400 mμ (11).

Because splenectomy in some patients has resulted in disappearance of anemia and diminution in photosensitivity and in porphyrin excretion, it is believed that the erythropoietic stimulus produced by the hemolytic anemia results in a marked augmentation of porphyrin production. The photosensitivity is a manifestation of the well-known photodynamic effect of porphyrins, described first by Hausmann (12).

Acute Intermittent Porphyria. The freshly passed urine is generally normal in color since it contains chiefly (colorless) porphobilinogen. On standing or heating in dilute acid this is converted to porphyrin, imparting to the urine a dark red-brown color.

The mechanism of the clinical disturbances is unknown. Earlier studies on production of smooth muscle spasm by porphyrins have recently been questioned by Goldberg and co-workers (13). The condition is inherited as a Mendelian dominant trait.

Other Forms of Porphyria. Mixed forms of hepatic porphyria are sometimes seen, with both photosensitivity and abdominal colic and/or neuropsychiatric disturbances. Porphobilinogen is demonstrable in the urine.

Individuals with latent porphyria are generally found among relatives of patients with acute intermittent porphyria. They show moderate increases in urinary porphobilinogen and porphyrins and have only minor or no pertinent clinical complaints.

EXPERIMENTAL PORPHYRIA

During the past few years we have succeeded in producing in rats and/or rabbits two types of so-called 'experimental porphyria' which have many features in common with the human disease.

An erythropoietic type of porphyria is produced in rabbits by first administering repeated doses of phenylhydrazine to produce a hemolytic anemia and to stimulate erythropoiesis (14). Lead

TABLE 3. CHEMICAL CHANGES IN RATS WITH SEDORMID PORPHYRIA

Substance	Units	Day 0	Day 3	Day 10
		conc. (approx.)		
From liver				
Copro	μ g%	10	80	100
Uro*	μg %	trace	30	1000
Porphobilinogen	μg/gm	"O"	"O"	100
Catalase	Kat F/gm	100	20	3
Green porphyrins	u/gm†	trace	40	40
Phospholipid	μg P/100 mg	120	170	200
Total lipid	mg/gm	50	75	120
Org. acid sol. P	μg/100 mg	90	60	45
From urine				
Copro	μg/day	20	80	100
Uro	μg/day	trace	15	500
Porphobilinogen	μg/day	"O"	trace	++++‡
From bile§				
Copro	μg %			20,000
Proto	μg %			40,000
Uro	μg %			1,000
From bone marrow				
Copro	μg %	5		5
Proto	μg %	40		40
Uro	μg %	trace		trace

No significant change in liver cytochrome oxidase, succinic dehydrogenase, ribo- or desoxyribonucleic acid, or phosphoprotein. Cytochrome *c* concentration seems to decrease only in relation to weight loss. The phospholipid, total lipid, and organic acid soluble P have not yet been fractionated further.

* All uroporphyrin analyses done after boiling tissue, urine, or bile extract.

† 1 unit has optical density at 400 mμ equal to that of 1 μgm of coproporphyrin.

‡ No accurate quantitative data available.

§ Data available only on rabbits sacrificed after approximately two weeks or more Sedormid feeding.

acetate is then injected, and the animal is exposed to unfiltered light from a mercury lamp. This is followed in the next day or two by a marked increase in the bone marrow and urinary uro- and coproporphyrin. Liver porphyrin concentration remains unaltered. Unlike the human disease, urinary porphobilinogen concentration is greatly increased.

A hepatic type of porphyria in rats and rabbits is produced by the daily oral administration of allyl-isopropyl-acetyl-carbamide (Sedormid) (15). A number of analogues have recently been shown by Goldberg and Rimington (16) to produce similar effects. These all contain the allyl group, but vary in the alkyl and acid amide or ureide substituent. The water-soluble allyl-isopropyl-acetamide introduced by them has been very useful since it can be administered parenterally and is non-hypnotic. Talman and her colleagues (17) have reported especially interesting results in chickens following injection of these compounds into the developing egg. A similar type of experimental porphyria also follows the administration of trional (diethylsulfoneethylmethyl-methane).

Chemical studies of the tissues and excreta of the Sedormid-treated animals are summarized in table 3. The most striking changes noted to date are these:

Porphyrins (uro-, copro-, and proto-) and porphobilinogen. The concentration of liver coproporphyrin rises rapidly to peak values in 2–3 days, and remains fairly constant thereafter. Porphobilinogen and uroporphyrin concentration rise only after several days. Before death rabbits may excrete 50 mg or more of porphobilinogen per day. Quantitative data on liver protoporphyrin is subject to considerable error because of contamination with green porphyrins (see below). Its liver concentration rises only slightly, though its concentration in bile is very high. The normal values found for bone marrow (and circulating erythrocytes) are similar to the finding in human hepatic porphyria.

Catalase. The enzymatic activity of liver catalase falls about 50% per day (18). In Sedormid-treated rats no significant C^{14} activity was found in liver catalase 2 days after the administration of glycine-2-C^{14}, while significant activity was found in the liver catalase of control normal rats. These studies suggest that Sedormid blocks the synthesis of liver catalase, and that the observed rate of decline of catalase activity reflects a 'half-life' of this substance of one day or less. The nature of this synthetic block is unknown. If Sedormid feeding is discontinued, liver catalase activity returns to normal in approximately [illegible] week.

Green porphyrins (6): Within a few days, the livers of Sedormid-treated animals became olive-green in color. (This is seen best after saline perfusion of the liver.) Considerable amounts of green pigment can be extracted from the liver and separated from other porphyrins and hemes by the procedure outlined in table 4. Six hours after the intraperitoneal injection of the acetamide compound, the concentration of green pigments increases at least 8-fold. During the first 2–3 days its concentration is 10–20 times that of uro-, copro-, and protoporphyrin combined.

Despite considerable effort it has not yet been possible to obtain this substance in crystalline form. Some of its properties, however, may be summarized as follows:

FRACTIONATION: It can be fractionated by various types of column and paper chromatography into several distinct pigments. Most are green. Others are some shade of brown or yellow.

FLUORESCENCE: Some fractions fluoresce in

tensely red when exposed to near ultraviolet light; others are non-fluorescent.

ABSORPTION: The absorption spectra of these compounds are similar to those of known porphyrins (especially chlorins and phorbins) but are identical with none. They are also clearly different from those of known green hemins, bile pigments, etc. All show Soret type absorption bands whose maxima range from about 380–430 mμ. The optical densities of this band are approximately 10–13 times that of the main band in the visible spectrum. The absorption maximum of the latter is shifted far into the red, being slightly over 700 mμ in some fractions. The infra red absorption spectrum is similar to that of other porphyrins (19) but is distinguished mainly by the unusually pronounced absorption at about 3300 cm^{-1}.

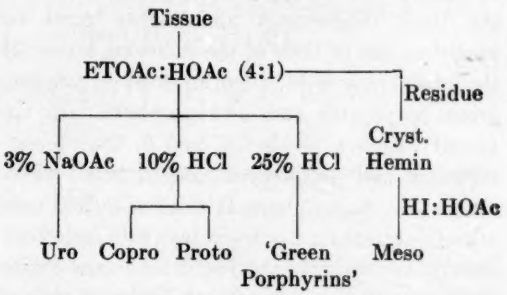
BIOSYNTHESIS: *a*) From glycine-2-C^{14}. Glycine-2-C^{14} was administered to 16 Sedormid-treated rats. They were killed in groups of 4 at various time intervals up to 68 hours. The specific activity curves of the coproporphyrin, protoporphyrin, and green porphyrins isolated from the pooled livers were similar, with maximum specific activities found in all within a few hours after the glycine administration. From the similar metabolic pattern of these compounds it was concluded that the green porphyrins cannot be derived from preexisting heme compounds such as catalase or hemoglobin.

b) From uro- and coproporphyrin. In the earlier described studies on the fate of uro- and coproporphyrin C^{14} in various biological systems it was noted that up to 10–15% of the activity employed could be recovered in green porphyrins separated by calcium carbonate chromatography. These C^{14} labeled compounds appear to be similar to the green porphyrins isolated from livers of Sedormid-treated animals, but further study is necessary to establish their identity.

METABOLISM OF PORPHOBILINOGEN

As noted above, the excretion of excessive amounts of porphobilinogen constitutes a basic abnormality in Sedormid-treated animals and in patients with acute intermittent porphyria. This might conceivably be due either to a block in its further metabolism or to its production at a rate which exceeds the capacity of the liver to metabolize it. The latter has recently been shown to be true (20). Liver homogenates from normal rats were incubated with added porphobilinogen while those from rats treated with Sedormid for 2, 5, and 13 days respectively were incubated both with and without the addition of porphobilinogen. The content of porphobilinogen and of porphyrins in the homogenates were determined at various time intervals. Results were similar in all cases regardless of the initial porphobilinogen concentration (91–383 μg/g of liver). From 47–67 μg of porphobilinogen disappeared per gram of liver during the first 5 hours from homogenates of normal rat liver, while the corresponding values from Sedormid rat livers ranged from 62 to 80 μg. During the same time interval porphyrin production was similar in all groups.

TABLE 4. PRELIMINARY TISSUE PORPHYRIN ANALYSIS: SCHEMATIC SUMMARY



To identify the porphyrins precisely, the above fractions are esterified with a 19:1 mixture of MeOH:H_2SO_4 for purification by column chromatography and crystallization.

DISCUSSION

Clinical studies of porphyria patients and the isolation of porphyrins and porphobilinogen from their excreta have been of paramount importance in stimulating chemical and biochemical studies of porphyrin metabolism. There is no doubt that the growing body of data being accumulated by the biochemist will soon have a corresponding impact on the clinical understanding of this disease. To date, however, this is insufficient to answer four key questions raised by the clinician:

1) Do the findings in experimental animals correspond to those seen in human patients with acute intermittent porphyria? The porphyrin and porphobilinogen pattern in tissues and excreta of patients and experimental animals appear to be similar in all respects studied to date. There is, however, no known data available on catalase activity or concentrations of other compounds listed in table 3 from livers of acutely ill patients. Fenton (addendum to ref. 16) has reported the absence of nerve demyelinization which is characteristically found in the human disease.

2) What is the basic biochemical abnormality in this disease? It appears at the moment that the basic biochemical abnormality must be sought at one or both of the following levels: *a*) the tetrapyrrole level, where an effect on catalase, green porphyrins and coproporphyrin are apparent within a few hours, and *b*) the porphobilinogen and pre-porphobilinogen level, where observable changes have thus far appeared only after treatment for 2 or more days with Sedormid. Shemin and Russel have found that delta-amino levulinic acid is a common precursor not only of porphobilinogen but also of purines and other substances as well (21). The suggestion has therefore been made (22) that a block in one of the other metabolic pathways of this compound may lead to its piling up and excessive conversion to pyrrol compounds. If such a mechanism does operate, it is not clear how this would result in the observed block of catalase synthesis, though it is conceivable that the same or related enzyme systems might be involved. The possibility also exists that the primary block involves conversion of porphyrins to heme enzymes, and the increased pyrrol synthesis results from an attempt by the liver to compensate for this block. This would be analogous to the stimulation of erythropoiesis produced in hemolytic or blood loss anemia.

3) What is the mechanism of production of the symptoms observed? It would now appear that porphobilinogen and porphyrins per se are not responsible for the symptoms of acute intermittent porphyria. While there is no experimental data on the subject, it is suggested that deficiencies of heme enzymes and/or other compounds in the nervous system may be the most likely cause of these symptoms.

4) What specific therapy can be offered the patient? ACTH and a large number of vitamins and other substances have proven ineffectual in the treatment of Sedormid porphyria (18). Many of the compounds tested have been recommended for the human disease, but all are of limited value. If the many compounds which induce porphyria act as competitive antagonists, one might expect suggestions for rational therapy to follow from studies of their molecular structure.

REFERENCES

1. Shemin, D. and J. Wittenberg. *Ciba Foundation Conf. on Isotopes in Biochemistry* 1951, p. 41.
2. Westall, R. G. *Nature* 170: 614, 1952.
3. Falk, J. E., E. I. B. Dresel and C. Rimington. *Nature* 172: 292, 1953.
4. Bogorad, L. and S. Granick. *Proc. Nat. Acad. Sci.* 39: 1176, 1953.
5. Schwartz, S., M. Keprios, M. Berg and C. J. Watson. To be published.
6. Schwartz, S. and K. Ikeda. *Ciba Conf. on Porphyrin Biosynthesis and Metabolism.* In press.
7. Granick, S. *Federation Proc.* 13: 219, 1954.
8. Schwartz, S. and H. M. Wikoff. *J. Biol. Chem.* 194: 563, 1952.
8a. Watson, C. J. *Arch. Int. Med.* 86: 797, 1950.
9. Zieve, L., H. Hill, S. Schwartz and C. J. Watson. *J. Lab. & Clin. Med.* 41: 663, 1953.
10. Schmid, R., S. Schwartz and C. J. Watson. *Arch. Int. Med.* 93: 167, 1954.
11. Schmid, R., S. Schwartz and R. D. Sundberg. *Blood* 10: 416, 1955.
12. Hausmann, W. *Biochem. Ztschr.* 30: 276, 1910.
13. Goldberg, A., W. D. M. Paton and J. W. Thompson. *Brit. J. Pharmacol.* 9: 91, 1954.
14. Schwartz, S., M. Keprios and R. Schmid. *Proc. Soc. Exper. Biol. & Med.* 79: 463, 1952.
15. Schmid, R. and S. Schwartz. *Proc. Soc. Exper. Biol. & Med.* 81: 685, 1952.
16. Goldberg, A. and C. Rimington. *Proc. Roy. Soc. London Ser. B* 911: 257, 1955.
17. Talman, E. L., J. D. Case, R. A. Neve, F. Labbe and R. A. Aldrich. *J. Biol. Chem.* 212: 663, 1955.
18. Schmid, R., J. F. Figen and S. Schwartz. *J. Biol. Chem.* In press.
19. Falk, J. E. and J. B. Willis. *Aust. J. Sci. Res., Ser. A.*, 4: 579, 1951.
20. Merchante, A., B. L. Wajchenberg and S. Schwartz. To be published.
21. Shemin, D. and C. S. Russel. *J. Am. Chem. Soc.* 75: 4873, 1953.
22. Labbe, R., E. T. Talman and R. A. Aldrich. *Federation Proc.* 14: 779, 1955.

Reference is also made to the following books or review articles:

Lemberg, R. and J. W. Legge. *Hematin Compounds and Bile Pigments. Their Constitution, Metabolism, and Function.* New York: Interscience, 1949. Waldenström, J. *Acta Med. Scandinav. Supp.* 82, 1937. Watson, C. J. and E. Larson. *Physiol. Rev.* 27: 478, 1947. Fischer, H. and H. Orth. *Die Chemie des Pyrrols.* Leipzig: Verlagsgesellschaft, 1937. Rimington, C. *Acta Med. Scandinav.* 143: 161, 1952. Vanotti, A. *Porphrins, Their Biological and Chemical Importance.* London: Hilger and Watts, 1954. Watson, C. J. *Advances Int. Med.* 6: 235, 1954.

INVESTIGATION OF DISEASES OF THE THYROID GLAND BY MEANS OF RADIOACTIVE IODINE

CHARLES A. OWEN, JR., WILLIAM M. MCCONAHEY, F. RAYMOND KEATING, JR. AND ALAN L. ORVIS

From the Sections of Clinical Pathology, Medicine, and Biophysics and Biophysical Research, Mayo Clinic and Mayo Foundation,[1] Rochester, Minnesota

SEVERAL large series (1–3) of patients with thyroidal diseases have been analyzed with the aid of radioactive iodine (I^{131}). The conclusion of these and other investigators has been that the addition of tracer tests utilizing I^{131} to existing laboratory procedures, such as the basal metabolic rate and determination of protein-bound serum iodine, has significantly increased the laboratory's usefulness with regard to evaluation of abnormalities of the thyroid gland.

Because of its penetrating gamma rays, I^{131} can be measured *in vivo* as well as *in vitro*. This has led to two basic types of tests of thyroid function using radioiodine, namely measurement of the rate or amount of thyroidal accumulation of the isotope and determination of the rate or amount of labeled hormone leaving the gland. Innumerable direct and indirect procedures have been devised to measure these functions.

PRESENT STUDY

It was decided to study a series of eight tests employing I^{131} in persons who did not have evidence of disease of the thyroid, as well as in groups of patients who had various disturbances of thyroidal function. Diagnoses were established on clinical grounds, taking into account the basal metabolic rates and values for protein-bound serum iodine. The data from the I^{131} tests were excluded from diagnostic consideration.

The study involved 469 persons, although no single I^{131} test was performed in all instances. Of this group, 46 were excluded from the series because of the recent use of drugs that invalidated the tracer studies (31 patients) or because a clear-cut diagnosis could not be established (15 patients). The control group in our series consisted of 181 patients and volunteers without any complaints referable to the thyroid gland and in whom clinical findings of thyroidal abnormality were absent. Exophthalmic goiter was diagnosed in 92 patients, nodular goiter in 65 and myxedema in 13. The remaining 72 patients included several small groups who had miscellaneous pathologic states of the thyroid; these groups will be considered elsewhere (4).

METHODS

For most patients the following procedure was used: 50 μc of NaI^{131} (carrier-free)[2] was administered intravenously; a continuous record was then made of the thyroidal uptake of I^{131} for the first half hour. Urine was collected at the conclusion of the half-hour test and fractionally during the next 2 days. The thyroidal uptake of I^{131} at 6 and 24 hours was determined. Finally, 24 hours after the dose was given, a sample of blood was drawn for assay of serum I^{131}.

Method 1: 6-Hour Thyroidal Uptake. Six hours after administration of a dose of I^{131}, the amount in the thyroidal region of the neck was measured. Sodium iodide (thallium-activated) scintillation crystals were used at a skin-crystal distance of 20 cm. With our 2.5-cm crystal (2.5 cm in diameter, 1.3 cm in length), the attenuator-discriminator settings were such that 1 μc of I^{131} registered 18 counts per second (cps) over a background of about 1 cps; with our 3.8-cm crystal (3.8 cm in diameter, 2.5 cm in length), the value was 80 cps, with a background of about 5 cps. The collimation of both counters was such that the surface of the neck 'viewed' by the crystals was a circle with a diameter of 8 cm.

Method 2: 24-Hour Thyroidal Uptake. This was like *method 1* except that the thyroidal uptake was measured 23–25 hours after administration of the dose.

Method 3: 24-Hour Urinary Excretion. Urine was collected fractionally for other purposes (see

[1] The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.

[2] Obtained from Abbott Laboratories under authorization of the Atomic Energy Commission.

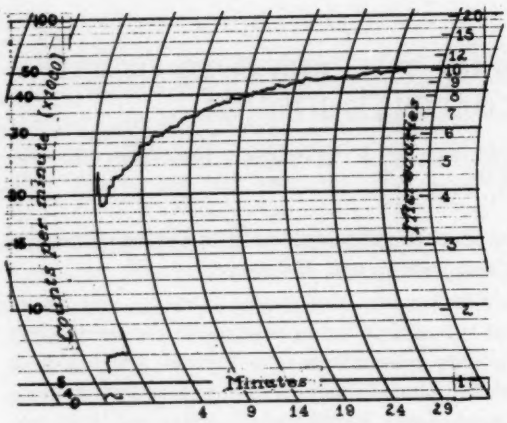


FIG. 1. Continuous recording of thyroidal uptake of I^{131} after intravenous administration of 20 μc. Thyroidal clearance of plasma I^{131} was calculated from this curve to be 187 ml/min.

method 4) during the first 24 hours. Aliquots of each sample were counted in a Texas Co. well counter of the Geiger-Müller type. The counter had a sensitivity of 870 cps per μc over a background of about 7 cps. Samples (60 ml) were compared with a diluted aliquot of a 'pilot' representing the administered dose. This technic avoided any need for absolute standardization of the counter, as well as obviating corrections for decay.

Methods 4 and 5: Extrarenal Disposal Rate and Thyroidal Accumulation Rate. The technics used were those developed by Keating and associates (5). The fraction of the administered dose of I^{131} appearing in the urine was determined at intervals of 2, 4, 6, 12, 18, 24 and 48 hours. The cumulative excretions were plotted as the fraction of the dose excreted versus time. From the final portion of the curve, an asymptotic value was assumed. The difference between any observed value and the asymptote was replotted logarithmically versus time. The slope of the resulting straight line is a measure of the rate of disappearance of I^{131} from the iodide space of the body. Essentially, the rate is the sum of two functions, namely renal excretion and thyroidal accumulation. By multiplying the rate of disappearance by the thyroidal uptake, one has a measure of 'thyroidal accumulation rate'; when the rate of disappearance is multiplied by the fraction of the dose actually excreted in the urine, the product is the 'renal excretion rate.' The difference between the total rate of disappearance and the 'renal excretion rate' has been called the 'extrarenal disposal rate'; this is the rate of migration of I^{131} to all tissues other than the kidney and, in general, it correlates well with the 'thyroidal accumulation rate.'

Methods 6 and 7: Protein-Bound I^{131} (6) and Conversion Ratio (7). Twenty-four hours after administration of the dose of I^{131}, at the time of the *in vivo* measurement, a sample of blood was taken and permitted to clot. The serum was assayed for total radioactivity. The proteins were precipitated in the same tube by the Somogyi zinc

TABLE 1. RESULTS OF TESTS UTILIZING RADIOIODINE IN VARIOUS THYROIDAL STATES

Test	Euthyroid Controls* (181)		Patients* in Normal Range, %			
					Nodular goiter	
	Mean	Normal range	Exophthalmic goiter (92)	Myxedema (13)	With hyperthyroidism (19)	Without hyperthyroidism (46)
6-Hr. thyroidal uptake, % of dose	14.9	7.5–25.0	0	0	58.3	93.9
24-Hr. thyroidal uptake, % of dose	24.8	11.9–46.5	11.1	7.7	77.8	100.0
24-Hr. urinary excretion, % of dose	58.8	36.5–74.9	8.9	91.7	56.3	97.8
Thyroidal accumulation rate, % of dose/hr.	3.3	1.1–8.0	11.1	16.6	80.0	95.6
Extrarenal disposal rate, % of dose/hr.	4.3	1.4–8.4	6.7	58.3	73.3	80.0
Thyroidal clearance of plasma I^{131}, ml plasma/min.	8.2	0–26.0	0	100.0	68.8	95.4
24-Hr. protein-bound serum I^{131}, % of dose/l. serum	.064	.014–.167	33.3	100.0	75.0	100.0
24-Hr. conversion ratio†	20.3	2.5–46.3	4.8	100.0	58.8	97.6
Protein-bound I, μg/100 ml serum‡	5.1	3.4–7.6	3.9	0	12.2	100.0
BMR, % deviation from normal‡	−4	−20 to +16.0	+11.1	+15.4	+18.7	+93.5

* Figures in parentheses = number of patients. † ($PBI^{131} \times 100$) ÷ total serum I^{131}. ‡ These data on protein-bound iodine and the basal metabolic rate are given for reference. Since they provided a portion of the basis for diagnosis, they were not themselves being evaluated in this study.

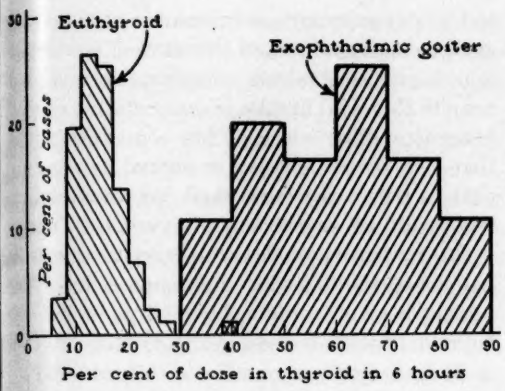


FIG. 2. Distribution of values for thyroidal uptake of I^{131} 6 hr. after administration of the dose. All the results in euthyroid persons and in those who had exophthalmic goiter are included. Only one normal value lies within the range of values for patients with exophthalmic goiter.

reagent and washed twice with water. The I^{131} of the precipitate was then assayed. The precipitated I^{131} was calculated both as a percentage of the administered dose per liter of serum (protein-bound I^{131}) and as a fraction of the total radioactivity of the serum (conversion ratio). For these measurements, a well-type sodium iodide (thallium-activated) crystal was employed; at our usual settings, it had a sensitivity of 16,000 cps per μc over a background of 4 cps.

Method 8: Thyroidal Clearance of I^{131} From the Blood. The method of Berson and colleagues (8) was used with two modifications, namely a 3.8-cm crystal was used for measurement of the I^{131} (as described under *method 1*) and a continuous tracing was made of the radioactivity during the half-hour period of observation instead of cumulative counts at intervals of 1 minute.

Figure 1 shows a characteristic pattern. The background over the patient's neck was about 7,000 counts per minute (cpm) from a previous tracer dose of I^{131}. After the intravenous administration of 20 μc of I^{131}, there was a rapid increase in activity over the thyroid (to 23,000 cpm), followed by the usual decrease as equilibrium between intravascular and extravascular iodide spaces became established. The equilibrial value was about 19,000 cpm, or 4 μc of I^{131}. The thyroidal value 30 minutes later was about 50,000 cpm, or 10 μc of I^{131}, a net gain of 6 μc, or 30 per cent of the administered dose. With the aid of the necessary additional data (height, weight and the half-hour urinary excretion), the clearance was calculated as 187 ml of plasma cleared of I^{131} per minute by the thyroid.

RESULTS

The results of the study are summarized in table 1. The total number of patients with each thyroidal abnormality is listed; the percentage of those subjected to each test whose values fell within the normal range is indicated for each diagnostic test.

The 'normal range' is defined for this study as the limits that included 95% of the values obtained in euthyroid persons; thus, we eliminated each group of values making up 2.5% of the total number and located at each extreme of the curve of distribution. This method was selected instead of the two-standard deviation technic because of the skewed distribution curves.

It can be seen that all the tests utilizing I^{131} separated, to a considerable extent, the euthyroid persons from patients who had exophthalmic goiter. Two of the methods, namely the thyroidal clearance and the 6-hour uptake, showed no overlap between the values for patients with exophthalmic goiter and values within the 95% range of

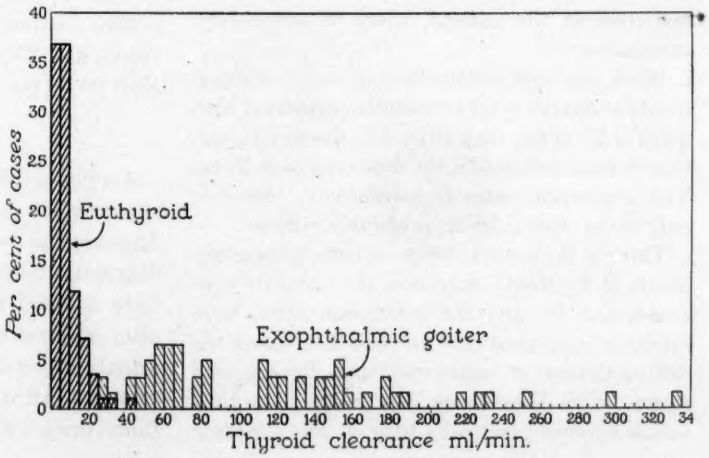


FIG. 3. Distribution of values for thyroidal clearance in normal persons and in patients with exophthalmic goiter. A slight overlap is seen in the vicinity of 30 ml of plasma cleared per minute.

normal. Figures 2 and 3 show the actual distribution of the patients' values and all control values with these two isotopic methods.

In the case of myxedema, only the values for 6-hour and 24-hour uptake were discriminating between the 95% range of normal values and those for myxedematous individuals. Again, the results of the 6-hour uptake were the more precise.

Tests employing I^{131} all fell short of being satisfactory in patients with nodular goiters; too many who had clinical hyperthyroidism exhibited normal values for I^{131}.

COMMENT

Several factors must be considered in the choice of I^{131} tests for the evaluation of thyroidal function; these include diagnostic precision, the patient's time and the laboratory time involved in completion of the test, the complexity of the method and the size of the dose of I^{131} required.

The briefest test is the thyroidal clearance, which requires about an hour from the time of injection until the result is calculated. The measurement of the 6-hour uptake is completed on the same day that the I^{131} is administered. The remainder of the tests necessarily carry over to the second day; this is also true of determination of protein-bound serum iodine.

The simplest tests involve oral administration of the I^{131} and measurement of uptake over the thyroid; such tests require only a few minutes of the patient's time. Urinary analyses are only slightly more time-consuming. However, calculation of the thyroidal clearance or of the thyroidal accumulation rate, or the performance of protein precipitation of serum, is definitely more tedious. Further, any urinary test requires complete cooperation of the patient, which is not always obtainable.

When one uses scintillation crystals, measurements of uptake are conveniently performed with doses of I^{131} of less than 10 μc. The thyroidal clearance is more accurate if the dose is at least 20 μc. The conversion ratio is satisfactory, however, only if the dose is 50 or, preferably, 100 μc.

Three of the isotopic technics, namely measurements of thyroidal clearance, the value for protein-bound I^{131} and the conversion ratio, have intrinsic limitations that prevent their use in the differentiation of euthyroid and hypothyroid states. With these tests, the range of normal values extends practically to zero. The clearance test in our experience is insensitive at the lower extreme and a calculated clearance of 0.0 ml per minute may be obtained, even though a small but definite thyroidal uptake is demonstrable several hours later. The extremely low conversion ratios that may be encountered in normal persons appear to reflect the exceedingly low level of protein-bound I^{131} occasionally observed at 24 hours.

Our evaluation of these various considerations has led to the following routine procedure. The patient is given orally a dose of 5 μc of I^{131}; the thyroidal uptake is measured 6 and 24 hours later. A 24-hour specimen of urine is collected and assayed for I^{131} for two reasons: *a*) as a check on the dose, since the sum of thyroidal uptake and urinary excretion in 24 hours is usually 80–90% of the dose, and *b*) a lagging urinary excretion of I^{131} often warns of cardiac or renal disability, which may lead to an artificially increased value for thyroidal uptake.

When these routine procedures yield equivocal results, a sample of blood is obtained for determination of protein-bound serum iodine (if the 24-hour uptake is less than 15% of the administered dose) or a thyroidal clearance is performed (if the 24-hour uptake is between 35 and 55% of the dose). Reviewing the records of the last 300 patients studied in this routine fashion, we found that 22.0% had values for the 24-hour uptake that were less than 15% of the dose, whereas 44.7% were between 15 and 34.9%, 17.3% were between 35 and 55%, and 16.0% were more than 55% of the dose. Thus, the study was concluded at the end of the 24-hour *in vivo* measurement in 60.7% of patients, whereas 22.0% were sent for determination of protein-bound iodine, and thyroidal clearance was performed in the remaining 17.3%. Of this last group (52 patients), 17 had normal clearances (5.7% of the entire 300 patients) and 35 (11.6%) had clearances of more than 26 ml per minute.

SUMMARY

A series of tests utilizing radioiodine was undertaken on 469 patients who had various thyroid diagnoses in order to evaluate clinically the diagnostic usefulness of these procedures. The tests included thyroidal uptake 6 and 24 hours after administration of a dose of I^{131}, 24-hour urinary excretion of I^{131}, thyroidal accumulation rate and extrarenal disposal rate based on fractional urinary assays for I^{131}, determination of the

protein-bound serum radioiodine and the conversion ratio 24 hours after administration of the dose, and measurement of the thyroidal clearance of plasma I^{131} (Berson method).

Ninety-two of the patients had exophthalmic goiter as determined by clinical evaluation correlated with the basal metabolic rates and the values for protein-bound iodine. In this group, the radioiodine tests generally, and particularly the 6-hour uptake and the thyroidal clearance tests, were excellent for differentiating overactive thyroidal function from normal states.

Thirteen patients were myxedematous. Of all the radioiodine tests, only the 6-hour uptake was a highly satisfactory method for this group. None of the radioiodine tests was of great help in differentiating hyperthyroidism from euthyroidism in 65 patients who had adenomatous goiters.

REFERENCES

1. Skanse, B. *Acta med. scandinav.* Suppl. 235: 1, 1949.
2. Werner, S. C., E. H. Quimby and C. Schmidt. *J. Clin. Endocrinol.* 9: 342, 1949.
3. Keating, F. R., Jr., S. F. Haines, M. H. Power and M. M. D. Williams. *J. Clin. Endocrinol.* 10: 1425, 1950.
4. McConahey, W. M., C. A. Owen and F. R. Keating, Jr. Unpublished data.
5. Keating, F. R., Jr., M. H. Power, J. Berkson and S. F. Haines. *J. Clin. Invest.* 26: 1138, 1947.
6. McConahey, W. M., F. R. Keating, Jr., and M. H. Power. *J. Clin. Invest.* 28: 191, 1949.
7. Clark, D. E., R. H. Moe and E. E. Adams. *Surgery* 26: 331, 1949.
8. Berson, S. A., R. S. Yalow, J. Sorrentino and B. Roswit. *J. Clin. Invest.* 36: 141, 1952

BIOCHEMICAL STUDIES IN RELATION TO COMPARATIVE SUSCEPTIBILITY TO EXPERIMENTAL ATHEROSCLEROSIS

LAURENCE O. PILGERAM

From the Department of Physiological Chemistry, School of Medicine, University of California, Berkeley, California, and the Department of Physiology, College of Medicine, University of Illinois, Chicago, Illinois

DURING the past few years many notable advances have been made in the study of atherosclerosis. Along with these advances it has become increasingly clear that a relationship exists between the metabolism of cholesterol and the development of this disease which exacts such a high toll from among our population each year. As a consequence, experimental cholesterol atherogenesis has been employed as one of the main tools for investigative studies. However, along with this approach, it has become obvious that there exists considerable variability among animal species in their susceptibility to experimental atherosclerosis. The work of Katz and associates (1), of Marx and co-workers (2), of Altschul (3) and the studies of Hueper (4) along with the studies of numerous other investigators indicate that the rat, for example, is relatively resistant to experimental atherogenesis, whereas the chick, rabbit, or the guinea pig are relatively susceptible. It would therefore appear to be of considerable importance to study in detail the biochemical differences which may be correlated with susceptibility or resistance to cholesterol atherogenesis.

Of primary importance is consideration of the ability of the species to metabolize cholesterol. Employing the cholesterol tolerance technique, Horlick, Feldman and Katz (5) have found that the disappearance time of intravenously injected cholesterol varied from 72 hours in the rabbit to 24 hours in the chick and to 12 hours in the rat. Also, it was reported (1) that the feeding of cholesterol to the chick induces a high degree of hypercholesterolemia, whereas proportional amounts fed to the rat have practically no effect. The studies of Cook *et al.* (6, 7) suggest that the ability of the guinea pig to metabolize cholesterol is about equivalent to that of the chicken. This demonstration of a correlation between species ability to resist atherogenesis and the apparent ability to metabolize cholesterol is of the highest significance, and serves as an excellent sign for the direction to be taken by future research endeavor. It would seem logical, therefore, that the road to follow would be a study of the biochemistry of cholesterol metabolism. An indirect way to study cholesterol metabolism may be to study phosphatide metabolism, for if phosphatide metabolism is correlated with atherosclerosis, as a survey of the literature will indicate, then it must also be correlated with cholesterol metabolism.

Thus it was demonstrated by Ladd, Kellner and Correll (8) in 1949 that the incidence and severity of cholesterol-induced atherosclerosis in rabbits is decreased if the blood phospholipid is elevated concomitantly with the cholesterol. They accomplished the increase in the phosphatides by employing surface active agents such as Tween 80 and Triton A20. In 1950 Gertler, Garn and Lerman (9) demonstrated that in the human being the ratio of plasma total cholesterol to phospholipid phosphorus was appreciably increased in coronary disease as compared with normal control groups. Both of these reports and numerous other papers indicate a relationship to exist between the phosphatides and the development of atherosclerosis in various species.

However, the nature of the biochemical mechanism by which the phosphatides are involved in the atherosclerotic process and possibly in cholesterol metabolism has remained obscure. The studies of Jones *et al.* (10) indicate that all the lipoprotein molecules from S_f 40,000 down to S_f 4, and possibly into the high density class, represent a sequence of molecules in a metabolic chain involved in the ultimate utilization of glyceryl esters and fatty acids. They summarized the available evidence indicating that components of high S_f value are progressively transformed into those of the lower S_f classes. Based on these tracer studies and on other studies, they arrived at the

further conclusion that the normal pattern is one showing no appreciable concentration of any lipoproteins above a certain limit (S_f 6) for the human. Deviating from this simple pattern there is a regular sequence of types of patterns which may be regarded as a progressive manifestation of the metabolic block occurring in the atherosclerotic.

Information giving some hint as to the biochemical nature of this metabolic block would be of the highest importance. In this regard Block, Barker and Mann in 1951 reported (11) a deficient clearing of the alimentary lipemia in atherosclerotic males as compared with the clearance rate in normal men and women. According to the observations of Gofman *et al.*, (12) the concentration of lipoprotein species with S_f values greater than 75 increases following fat-containing meals, while the species with S_f values between 30 and 70 constitute the major fraction of the alimentary lipemia and are greatly modified in concentration with relationship to meals. Thus it appears reasonable to assume that the deficiency in the clearing of the alimentary lipemia observed in the atherosclerotic by Block *et al.* represents part of the metabolic block which slows or inhibits the progressive transformation of the high S_f components into smaller low density lipoproteins or even into α_1 lipoproteins.

The observation of Block *et al.* provides some indication as to the possible nature of part of the metabolic block in the atherosclerotic when it is taken into consideration along with the results obtained by other investigators. In 1949 Ahrens and Kunkel (13) reported that if one introduced the alpha toxin of *clostridium welchii* into serum one could produce or increase the lipemic state. This observation brings into sharp focus the results obtained by Block *et al.*, since Macfarlane (14) and Zamecnik, Brewster and Lipmann (15) demonstrated that the alpha toxin is an enzyme which is specific for the degradation of phosphatidyl choline with a very slow degradation occurring for sphingomyelin. Phosphatidyl serine and phosphatidyl ethanolamine were shown to be unaffected. Collectively these results suggest that a part of the metabolic block found in the atherosclerotic is related to the system for the formation of phosphatidyl choline. Reinforcing this tentative conclusion is the discovery by McLean (16) of heparin in a mixture of phosphatides which provided evidence for the intimate biochemical relationship of these substances. This work has been supported by the studies of Anderson and Fawcett (17). These observations taken together with the initial observation by Hahn (18) of the involvement of heparin in a lipemia clearing system bring the studies of Block *et al.* and of Ahrens and Kunkel into a close and significant relationship.

In view of this correlation of the data occurring in the literature, it was of interest to determine if a correlation could be demonstrated between the ability of an animal species to form phosphatidyl choline from free ethanolamine and the resistance of the species to experimental atherosclerosis. As is indicated in table 1, there occurred a striking difference between the ability of the atherosclerotic resistant rat to form phosphatidyl choline from free ethanolamine and the ability of the relatively susceptible chick or guinea pig to accomplish this process (20). The chick and the guinea pig possess a total liver capacity which is equal to only 3–4% of the capacity of the rat. If the ratio of phosphatidyl choline formation of the liver to body weight in grams is considered, one discovers that the ability of the chick and the guinea pig to carry out the reaction falls to 1–2% of the ability of the rat.

The results obtained with ethanolamine-1,2-C^{14} could be interpreted as indicating that, instead of a greater capacity for synthesis, the rat liver possessed a larger metabolic pool of methionine for the methylation of ethanolamine than did the chick or the guinea pig, or that the rat liver diluted the labeled ethanolamine less than did the liver tissue of the other two species, with the consequence that a greater yield of labeled phosphatidyl choline was produced. That this is not correct is proven by the much higher radioactivity of the phosphatidyl choline of rat liver obtained when $C^{14}H_3$-methionine was used as the methyl donor for unlabeled ethanolamine.

The results of these experiments on a small number of species demonstrate for the first time a correlation between the activity of a specific endogenous biochemical reaction system and the resistance to experimental atherogenesis. The results support the conclusions derived from the correlation of data obtained from the literature, namely that a part of the metabolic block found in the atherosclerotic is concerned with the system for the formation of phosphatidyl choline. Also the fact that the ability to form phosphatidyl choline correlates with the apparent ability

TABLE 1. RELATIVE ABILITY OF NONFASTED LONG-EVANS STRAIN RATS, ENGLISH SHORTHAIR GUINEA PIGS AND WHITE LEGHORN CHICKS TO CONVERT ETHANOLAMINE-1,2-C^{14} TO PHOSPHATIDYL CHOLINE

Species	No.	Counts*/500 mg Liver	Counts*/ Total Liver	Total Liver Count/gm of Animal
Rat	10†	1,180	16,520	94.5
	2‡	32,000		
Guinea pig	4†	9	515	1.1
Chick	4†	40	640	2.1
	2‡	768		

Liver slices, totaling 500 mg, were incubated aerobically for 2 hr. at 37° C in 5 ml of Krebs-Ringer phosphate solution containing 0.0016M $CaCl_2$ at a pH of 7.4. The isolation of phosphatidyl choline was carried out as reported previously (19) with the exception that the acetone-precipitation step was eliminated and that choline was counted as the Reinecke salt derivative.

* Total counts in phosphatidyl choline/100 sec.

Substrates: † Ethanolamine-1,2-C^{14} (3.8×10^{-4} M and 239,000 counts/100 sec.); methionine (7.5×10^{-4} M). ‡ $C^{14}H_3$-methionine (3.0×10^{-4} M and 311,000 counts/100 sec.); ethanolamine (3.8×10^{-4} M).

to metabolize cholesterol merits further attention.

In view of these results it appeared of importance to determine what the effect of a high cholesterol diet would be upon the system in an animal highly resistant to cholesterol atherogenesis. In this regard we placed young litter mate male rats of the Long-Evans strain on diets consisting of cholesterol and cholesterol plus cholic acid for 14–21 days before they were killed. The livers were excised and liver slices prepared. These liver slices were incubated as indicated in table 1 in the presence of C^{14} labeled ethanolamine or methionine and the formation of phosphatidyl choline measured. The data presented in table 2 demonstrate that dietary cholesterol increases the yield of phosphatidyl choline in the *in vitro* rat liver system (21). Cholic acid is necessary for the reaction to occur. In the absence of cholic acid the reaction to dietary cholesterol was quite variable, with about as many negative results as positive. The role of cholic acid may be one of increasing absorption of cholesterol from the rat gastrointestinal tract. This is indicated by the work of Swell *et al.* (22), where it was found that the presence of 1% cholate in the diet caused a progressive increase in the concentration of blood cholesterol which continued up to 3 weeks. In a few of our experiments cholic acid was observed to increase the yield of phosphatidyl choline in the absence of cholesterol addition to the basic diet. This effect may be due to an increased absorption of the normal dietary cholesterol, since the magnitude of the increase was not as great as it was when cholic acid and cholesterol were fed together.

The question arises as to the effect of cholesterol feeding upon the synthesis of phosphatidyl choline in other species such as the chick, guinea pig, or the rabbit. In view of the relative inability of the chick or the guinea pig to form phosphatidyl choline from ethanolamine it is reasonable to assume that even if cholesterol feeding were to increase the yield of phosphatidyl choline in these species, nevertheless the total amount of the phosphatide formed from ethanolamine would still be insignificant as compared to the amount formed by the rat.

The observation and interpretation of the preceding results merit special consideration in view of results in the literature. Namely, Perlman and Chaikoff (63) reported that the feeding of cholesterol for 30 hours decreases phospholipid turnover in the liver of rats as measured by P^{32}. However, they also found that choline stimulated phospholipid turnover and that in the presence of cholesterol and choline the activity of P^{32} labeled phosphatides was approximately three times as high as when cholesterol alone was fed. In view of the conditions of their experiments, it is necessary to point out that in our experiments *a*) the rats were fed cholesterol for periods ranging between 14 and 21 days; *b*) the metabolic study was carried out *in vitro*; *c*) the necessary substrates for the formation of choline were present; and *d*) our studies concerned a carbon chain moiety of the phosphatide molecule which may follow a metabolic pathway and possess a rate of turnover quite distinct from the phosphorus moiety. Of interest in this regard is the observation of Cornatzer (24) that the rat possessed a capacity for the formation of P^{32} labeled phosphatides which was about four times as great as that of the guinea pig upon the basis of specific activity. In our experiments with C^{14} labeled ethanolamine we found the capacity of the rat to exceed that of the guinea pig by about 32 times upon the basis of total liver activity. The

TABLE 2. EFFECT OF CHOLESTEROL AND CHOLIC ACID ON PHOSPHATIDYL CHOLINE FORMATION

Cholesterol in Diet	Cholic Acid in Diet	Basic Diet	No. Expt. Results	Av Liver Wt., gm	Liver	Initial Av. Wt. of Animals, gm	Final Av. wt. of Animals, gm	Total Counts in Phosphatidyl Choline/100 sec./500 mg Tissue	Total Liver Count*/100 sec.
−	−	+	8†	8.4	Normal	89	186	798	13,406
−	+	+	3†		Normal	90	188	889	
+	+	+	8†	10.6	Fatty	89	156	1040	22,048
−	−	+	2‡	9.3	Normal	92	194	3182	59,185
+	+	+	2‡	11.8	Fatty	88	182	4561	107,640

* Total liver count = (liver wt. in mg/500) × (phosphatidyl choline count/100 sec.).
Substrates:
† Ethanolamine-1,2-C^{14} (3.8×10^{-4} M and 239,000 ct/100 sec.); methionine (7.5×10^{-4} M).
‡ $C^{14}H$-methionine (3.0×10^{-4} M and 311,000 ct/100 sec.); ethanolamine (3.8×10^{-4} M).

explanation for the difference in these results may be due to different metabolic pathways or to the relative inability of the guinea pig to form choline from ethanolamine.

Our results suggest that the rabbit, being closely related to the guinea pig in dietary habits and closely related to the chick in susceptibility to cholesterol-induced atherogenesis, would react much the same as anticipated for the chick and the guinea pig in the formation of phosphatidyl choline from ethanolamine in the presence of dietary cholesterol. However, the results obtained with P^{32} by Zilversmit, Shore and Ackerman (25) indicate that the rabbit does respond to cholesterol feeding with a considerable increase in liver phosphatide synthesis. On the other hand, Marfori, Vulterini and Boccardelli (26) have observed that the administration of cholesterol to rabbits caused a decrease in the phosphatides of the liver. If they administered choline or inositol simultaneously with the diet, they were able to prevent the increase in liver cholesterol and the decrease in the liver phosphatides for 53 days. Choline was more effective than inositol. Both of these results may be reconciled if one assumes that cholesterol metabolism requires the presence of phosphatides. Thus even though cholesterol feeding stimulates the formation of phosphatides, the use of the phosphatide from the liver may exceed the rate of synthesis.

In order to reconcile all the observations, one may postulate that the essential difference between the different species lies in the inability of atherosclerotic susceptible animals to convert ethanolamine to choline, or, possibly, to convert phosphatidyl ethanolamine to phosphatidyl choline. Additional evidence supporting this conclusion or implicating the reaction system in atherosclerosis may be summarized as follows. Jukes has found that chickens could use monomethylethanolamine but not ethanolamine for growth and prevention of perosis (27, 28). The feeding of a diet deficient in methionine, the amino acid which is necessary for choline formation, but containing cholesterol has been found by Mann *et al.* (29) to produce atherosclerotic lesions in monkeys. This observation merits considerable attention, since here one is dealing with the primates which includes man. It has also been known for some time that thyroxin plays an important role in the atherosclerotic process. For example, considerable difficulty was encountered in making the dog atherosclerotic until Steiner and Kendall (30) produced atherosclerosis in this species by cholesterol feeding and antithyroid drugs. It is therefore of considerable interest to note that thyroxin has been found effective as a lipotropic agent against hepatic cholesterosis in contrast to the ineffectiveness of choline. However, Forbes (31) has found that choline is necessary in order for thyroxin to be effective.

Two other observations which add to the complexity of the problem but nevertheless implicate the ethanolamine-choline system are the studies of Rinehart and Greenberg (32) where they demonstrated that pyridoxine-deficient monkeys develop arteriosclerotic lesions, and the very recent studies reported at the American Federation of Clinical Research by Davis, Oester and Friedman where they demonstrated a decrease in the incidence and the severity of cholesterol-induced atherosclerosis in adenosinetriphosphate treated rabbits. In reference to the former ob-

servation it is known that pyridoxine is necessary for the action of decarboxylase, an enzyme presumably responsible for the formation of ethanolamine from serine (33, 34). The latter observation suggests the involvement of the ethanolamine-choline system in the atherosclerotic process in view of the report by Cantoni (35) that adenosinetriphosphate is necessary for the activation of methionine via S-adenosyl methionine.

Grateful acknowledgment is made to Professor David M. Greenberg for his kindly interest and encouragement in this problem.

REFERENCES

1. Katz, L. N. and J. Stamler. *Experimental Atherosclerosis*. Springfield, Ill.: Thomas, 1953.
2. Marx, W., L. Marx, E. R. Meserve, F. Shimoda and H. J. Deuel, Jr. *Arch. Pathol.* 47: 440, 1949.
3. Altschul, R. *Selected Studies on Arteriosclerosis*. Springfield, Ill.: Thomas, 1950; *Am. Heart J.* 40: 401, 1950.
4. Hueper, W. C. *Arch. Pathol.* 38: 162, 245 and 350, 1944; 39: 51, 117 and 187, 1945.
5. Horlick, L., M. Feldman, Jr. and L. N. Katz. *Proc. Soc. Exper. Biol. & Med.* 68: 243, 1948.
6. Cook, R. P. and G. P. McCullagh. *Quart. J. Exper. Physiol.* 29: 283, 1939.
7. Cook, R. P. and R. O. Thomson. *Biochem. J.* 49: 72, 1951.
8. Ladd, A. T., A. Kellner and J. W. Correll. *Federation Proc.* 8: 360, 1949.
9. Gertler, M. M., S. M. Garn and J. Lerman. *Circulation* 2: 205, 1950.
10. Jones, H. B., J. W. Gofman, F. T. Lindgren, T. P. Lyon, D. M. Graham, B. Strisower and A. V. Nichols. *Am. J. Med.* 11: 358, 1951.
11. Block, W. J., N. W. Barker and F. D. Mann. *Circulation* 4: 674, 1951.
12. Gofman, J. W., H. B. Jones, F. T. Lindgren, T. P. Lyon, H. A. Elliott and B. Strisower. *Circulation* 2: 161, 1950.
13. Ahrens, E. H., Jr. and H. G. Kunkel. *J. Exper. Med.* 90: 409, 1949.
14. MacFarlane, M. G. *Biochem. J.* 42: 587, 1948.
15. Zamecnik, P. C., L. E. Brewster and F. Lipmann. *J. Exper. Med.* 85: 381, 1947.
16. McLean, J. *Am. J. Physiol.* 41: 250, 1916.
17. Anderson, N. G. and B. Fawcett. *Proc. Soc. Exper. Biol. & Med.* 74: 768, 1950.
18. Hahn, P. F. *Science* 98: 19, 1943.
19. Pilgeram, L. O., E. M. Gal, E. N. Sassenrath and D. M. Greenberg. *J. Biol. Chem.* 204: 367, 1953.
20. Pilgeram, L. O. and D. M. Greenberg. *Science* 120: 760, 1954.
21. Pilgeram, L. O. and D. M. Greenberg, *Circ. Research* 3: 47, 1955.
22. Swell, L., D. F. Flick, H. Field, Jr. and C. R. Treadwell. *Proc. Soc. Exper. Biol. & Med.* 84: 428, 1953.
23. Perlman, I. and I. L. Chaikoff. *J. Biol. Chem.* 128: 735, 1939.
24. Cornatzer, W. E. *Ann. New York Acad. Sci.* 57: 919, 1954.
25. Zilversmit, D. B., M. L. Shore and R. F. Ackerman. *Circulation* 9: 581, 1954.
26. Marfori, L., S. Vulterini and V. Boccardelli. *Rass. fisiopatol. clin. e terap.* 22: 447, 1950.
27. Jukes, T. H. *J. Nutrition* 22: 315, 1941.
28. Jukes, T. H., J. J. Oleson and A. C. Dornbush. *J. Nutrition* 30: 219, 1945.
29. Mann, G. V., S. B. Andrus, A. McNally and F. J. Stare. *J. Exper. Med.* 98: 195, 1953.
30. Steiner, A. and F. E. Kendall. *Arch. Path.* 42: 433, 1946.
31. Forbes, J. C. *Endocrinology* 35: 126, 1944.
32. Rinehart, J. F. and L. D. Greenberg. *Am. J. Path.* 25: 481, 1949.
33. Stetten, D., Jr. *J. Biol. Chem.* 140: 143, 1941.
34. Arnstein, H. R. V. *Biochem. J.* 48: 27, 1951.
35. Cantoni, G. L. *J. Am. Chem. Soc.* 74: 2942, 1952.

CLINICAL CHEMICAL SIGNIFICANCE OF IONOGRAPHY

HUGH J. MCDONALD, EDWARD W. BERMES, JR. AND ROBERT H. SPITZER

From the Department of Biochemistry, The Graduate School and Stritch School of Medicine, Loyola University, Chicago, Illinois

ALTHOUGH the technique of separating mixtures of substances on the basis of differences in their rates of electromigration through stabilized media can be traced back, in a frequently broken chain, for about 70 years, its wide-spread development and application has occurred largely since 1950 (1). The number of published papers and reports, now exceeding a thousand, on various applications, modifications and limitations of the technique has grown so phenomenally since that time that this simple procedure has moved into the front ranks of important tools in biochemical and clinical chemical research. The technique of resolving mixtures of compounds on the basis of the relative differences in their electromigration rates through media stabilized with filter paper and other materials such as agar, starch, glass cloth, etc., was developed independently in several laboratories. It is only natural, then, that this fact should be reflected in the nomenclature used to describe the technique and associated operations or records. In this report the term 'ionography' will be used to describe the electromigration of all types of migrants, both colloidal and ionic, in stabilized media. The term 'ionogram' will be used to describe the end result from such experiments in which mixtures have been fractionated into discrete zones or areas, while the term 'ionographic apparatus' will refer to the instrument used for carrying out ionographic separations. Since filter paper has been the most widely used stabilizer, it can be assumed in this article that it is the material used unless stated to the contrary.

PRINCIPLES AND DESIGN OF APPARATUS

Although many variations in the technique of conducting electromigration experiments in stabilized media have been described, the great majority can be classified on the basis of design, as follows: *1) Closed-strip design: a)* sandwich technique, *b)* solvent immersion. *2) Open-strip design: a)* vertical suspension, *b)* horizontal suspension, *c)* ridgepole suspension.

In the sandwich technique (2–4), the paper strip is clamped between two glass or plastic plates and is generally held in a horizontal position. In the solvent immersion technique (5, 6), the paper strip is immersed in a bath of liquid such as chlorobenzene or carbon tetrachloride or is overlaid with a hexane, heptane or similar non-water-miscible liquid. In the vertical arrangement, the paper strip is generally inclosed in a tall glass cylinder (7–9). The potential is impressed over the paper strip by placing one electrode in the trough holding the upper end of the strip and the other in the bottom of the cylinder. In the open-strip horizontal suspension arrangement (10–12), the paper ribbon is usually pulled taut in a framework, with the ends dipping into the buffer vessels which in turn are connected with electrode vessels by means of agar-filled salt bridges or have reversible-type electrodes immersed directly in them. A schematic diagram of an ionographic apparatus of this type is shown in figure 1. In the ridgepole suspension technique (13, 14) the paper strip is draped symmetrically over a raised glass rod or similar structure so as to form an inverted V, with the ends of the strip extending down into the buffer solution. The framework and strips are generally inclosed in a suitable plastic chamber.

Variations of the more basic designs include such arrangements as *a)* altering the sandwich arrangement by placing the moist paper strip or sheet in direct contact with a lower glass plate while separating it from the upper plate by a thin air space (15), *b)* altering the sandwich technique, or the open-strip, vertical or ridgepole techniques to provide for simultaneous combinations of electromigration and chromatography, where both processes play an approximately equal role in the separation processes (16–20). Where paper or similar materials are used, this arrangement is sometimes referred to as 'curtain electrophoresis,' although the use of the word 'electrochromatography,' a term originally suggested by Berraz (8) would seem more appropriate, *c)* adaptations of the ridgepole technique where evaporation of solvent from the strip is actually promoted by providing perforations in the sides of the protecting shield over the paper strips (21, 22).

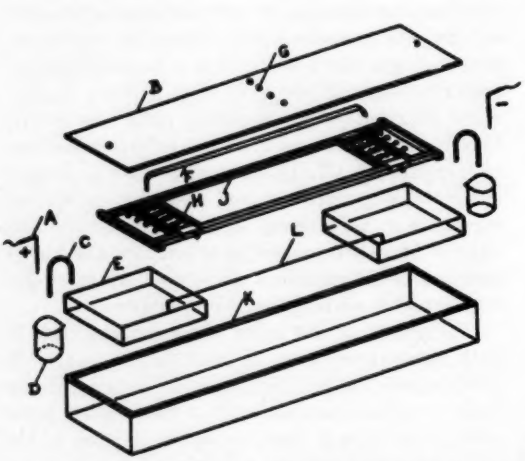
Where agar, starch or similar type stabilizers are used, stabilized media may be placed in hori-

zontal or vertical tubes, rolled in sheets of thin, pliable plastic film, placed between sheets of glass or plastic, or put in horizontal troughs, with the upper surface exposed to the solvent-saturated atmosphere in an inclosed space (1).

SOME APPLICATIONS IN CLINICAL CHEMISTRY

About 90% of the thousand or so papers which have been published on the technique of ionography deal with proteins, protein derivatives, protein hydrolytic products, lipoproteins, carbohydrates, enzymes, hormones and vitamins. Included among the papers dealing with proteins will be found procedures for the fractionation, identification and analysis of normal and pathological blood serum proteins, normal and abnormal hemoglobins, urinary proteins, cerebrospinal fluid proteins, iodinated proteins, gastric juice proteins, serum proteins of various animals, aqueous humor proteins, lens proteins, muscle proteins, antibodies, antigens, hemolysins, agglutinins, milk proteins, enzymes, protein hormones, peptides and amino acids (1, 23–26). This wide spectrum of applications to the protein and allied fields is in sharp contrast to the literature of chromatography, from which it is evident that only limited success has been attained in protein investigations. Some of the drawbacks associated with the chromatographic process include slow and incomplete resolution, difficult elution of proteins from columns and variable pH and salt effects that complicate the resolution.

That electrophoresis in non-stabilized media, i.e. so-called 'classical,' 'moving-boundary' or 'free-solution' electrophoresis, has proved to be an extremely valuable method for the separation and quantitative estimation of the proteins, is certainly evident from a perusal of the scientific literature. There are a number of factors, however, which limit its clinical use: good apparatus is not inexpensive, relatively large amounts of proteins are required, time-consuming manipulations are associated with the preparation of the electrophoresis cell, and the subsequent analytical



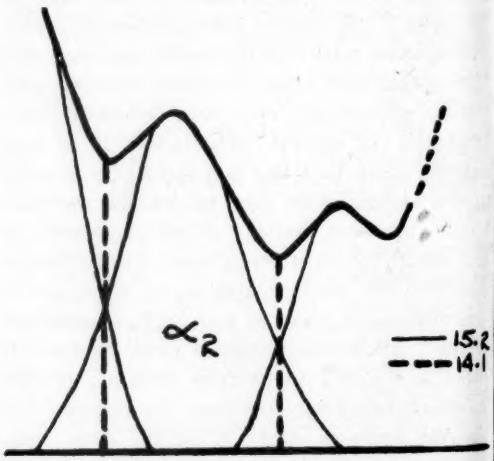


FIG. 1 (*left*). Schematic diagram illustrating the principal features of an ionographic apparatus of the horizontal open-strip type. Paper strip, *F*, is held horizontally in frame, *J*; the end clamps, *H*, containing the spring-actuated drawbolts for holding the paper strips are movable, thus allowing for variable length paper strips or sheets. The ends of the paper strips or sheets dip into the buffer solution contained in the buffer vessels, *E*. These, in turn, are connected by means of the inverted U-tubes, *C*, to the electrode vessels, *D*. The U-tubes are filled with agar-stabilized KCl solution. Platinum or Ag-AgCl electrodes, which are connected to a d.c.-regulated voltage source, dip into the electrode vessels. The solution level in each of the buffer vessels, *E*, is maintained at a constant height throughout a run, by a glass siphon tube, *L*, of small bore whose ends dip into the buffer solution. Except for the electrode vessels, *D*, and salt-bridges, *C*, all components of the apparatus are inclosed within the chamber *K*, which is covered by the lid, *B*. The migrant may be added to the strips by inserting a micropipet through the openings, *G*. The open space within the chamber is made minimal by proper design and by pouring water into the chamber, *K*, up to the level of the buffer vessels.

FIG. 2 (*right*). Two procedures for estimating areas under a curve, as typified by the alpha-2 globulin fraction of human plasma. *Dotted line*, perpendiculars dropped to base-line from troughs between peaks. *Solid line*, the curve is reflected, approximately, through the midpoint of the perpendiculars.

manipulations are complex. In addition to these factors, complete separation of two or more components is virtually impossible (26a) and the use of the method for the study of lipoproteins is difficult because they complicate the electrophoretic separation by giving rise to more components in an already multicomponent system and by rendering boundaries non-planar (27).

The proteins, being composed of amino acids and in some instances a small percentage of phosphates or other ionizable groups, exist in aqueous solutions as electrically charged entities. Other than amino acid end-groups, the specific amino acids and their groups responsible for ionization include arginine, the guanidinium group; aspartic acid, the beta carboxyl group; glutamic acid, the gamma carboxyl group; histidine, the imidazole group; lysine, the epsilon-amino group; tyrosine, the phenolic group; and iodinated tyrosine derivatives, also the phenolic groups. The magnitude of the electric charge on the protein is related to the number of these ionizable groups and to the environment of the protein. The degree of ionization depends on the pH, ionic strength of the medium, temperature and the presence of non-ionic organic compounds. Besides affecting the ionization of the aforementioned groups, the buffer ions of the solutions are capable of binding with the protein; for example, the isoelectric point of a given protein is dependent both upon the buffer system employed and upon the buffer concentration. This is due to the binding of buffer ions with the protein.

The electrophoretic mobility of a given protein is a function of the net electric charge and hence of the number of ionogenic groups, molecular weight and shape, and the environmental factors. By intelligent control of the variables, primarily pH, a mixture of proteins may usually be resolved with any method whose efficacy depends on differences in electromigration rates.

PRETREATMENT OF PROTEINS FOR IONOGRAPHIC ANALYSIS

Although it is unnecessary, in the qualitative ionographic resolution of plasma proteins, to dialyze the protein solution against the buffer solution through which the proteins are to migrate, preliminary dialysis does lead to cleaner-cut patterns on the ionograms, and in the determination of mobilities, it sometimes becomes a necessity. Graf and List (28), for example, observed a nonuniform and occasionally a reversed movement of horse serum proteins when the migrant was not previously dialyzed against the buffer. These effects were eliminated by dialysis. The theory and practical utility of dialysis are discussed by Clark (29), Holmes (30) and Stauffer (31).

Urinary and cerebrospinal fluid proteins are usually concentrated before ionographic separation. An increased protein concentration may be achieved by dialysis of the biological fluid containing the proteins against a fairly concentrated colloidal solution. Slater and Kunkel (32) dialyzed urine against a 25% aqueous solution of polyvinylpyrrolidone to concentrate the urinary proteins. Dextran (33) and gum acacia (34, 35) have also been employed. It must be emphasized however, that the indiscriminate use of such procedures can lead to errors. For example, should the molecules be of such size that they penetrate the dialysis membrane, complications can occur in subsequent analyses of the proteins. This type of error is illustrated by the case of polyvinylpyrrolidone, which contains nitrogen and is also capable of binding dyes. In such an instance, the determination of protein location and protein quantity by micro-Kjeldahl or dye-elution methods becomes quite complex.

Since the protein concentration in cerebrospinal fluid is much less than in blood plasma (0.016–0.038 gm % as compared to 6.5–7.8 gm %) a preliminary procedure for concentrating the proteins is generally necessary before ionographing the sample. Some of the methods which have been described include dialysis against high molecular weight materials such as dextran or polyvinyl-pyrrolidone (36), precipitation with acetone (37, 38), centrifugation and ultrafiltration (39) and ultrafiltration (40). The microbiuret method for protein determination described by Goa would seem to be applicable to cerebrospinal fluid studies as related to ionography (41).

In general, it has been found that body fluids containing not less than 2 grams of protein per 100 milliliters may be analyzed without preliminary concentration. Furthermore, for routine blood analyses, serum is preferable to plasma since the extra fibrinogen band complicates the ionogram, and the serum samples should be fresh and unhemolyzed. Adjutantis (42) found that success in resolving the soluble liver proteins of the rat is dependent on the freshness of the prepared material. Under optimal conditions five

major protein components were obtained (borate buffer, pH 8.6, ionic strength 0.18). If the protein solution was 'aged' for 48 hours, only one band could be demonstrated.

BUFFER SOLUTION

As regards the effect of the buffer solution on the rate of protein resolution, it has been observed that an increase in ionic strength generally causes the mobility of all protein components to decrease, but not necessarily at the same rate. At the extremes of the pH range the differences in the mobilities of all components will be greatest if the possibility of denaturation or adsorption effects on the stabilizer is precluded. Most workers who have studied proteins have chosen to conduct their investigations at a basic pH in the neighborhood of 8.6, in order to reduce adsorption effects to a minimum (43, 44). A veronal buffer having an ionic strength of approximately 0.05, is commonly employed. It may be prepared by weighing out 13.3 grams of sodium diethyl barbiturate and 1.84 grams of diethyl barbituric acid and adding water to make 1 liter of solution. Such a buffer solution is 0.05 molar with respect to the salt and 0.01 molar with respect to the acid. If the protein is to be determined by nitrogen analysis, a reagent blank must be obtained since nitrogen is a constituent of the buffer molecules.

A nomogram for acetate buffers has been assembled by Boyd (45). Gomori (46) has described the use of 2,4,6-collidine, tri(hydroxymethyl)-amino-methane, and 2-amino-2-methyl-1,3 propanediol, each of which when added to hydrochloric acid can be used as buffers in the general range of pH 6.5–9.6. Miller and Golder (47) have reported on a set of buffers of value in the pH range 2–12. Other buffer systems and their preparation include: phosphate (48–50), acetate (48), barbiturate (50), phthalate (49), borate (49) and veronal-acetate (51).

THE STABILIZER

Excellent protein fractionations have been obtained with such stabilizing agents as filter paper, agar and starch. In the case of paper, some of the factors which must be considered in making an appropriate choice include: *1*) the wet strength of the paper, the requirements in this respect for the horizontal openstrip and ridgepole techniques being different from those for the sandwich technique; *2*) filter paper thickness, since a hard, thick paper is usually preferred in the sandwich technique; *3*) impurities in the paper that may interfere in subsequent analytical procedures. (A filter paper blank is commonly run when micro-Kjeldahl determinations are called for.) *4*) The interaction between the paper and the dye used which may lead to difficulties in the dye-elution technique, e.g., Eaton-Dikeman #950 filter paper combines so strongly with bromphenol blue that the dye is eluted only with great difficulty.

Although paper has been used as the stabilizer in over 95% of the published articles on ionography, the use of starch has been found to have certain advantages in special cases. The pioneering work in determining the characteristics and potentialities of starch as a stabilizer has been done principally by Kunkel and his associates (24). One of the advantages claimed for starch is based on the lowered adsorption of serum proteins and lipoproteins as compared to paper. The technique, which includes both starch-block and column techniques, has found greater application in qualitative separations and small scale preparative procedures than in mobility determinations (24, 52–63).

Protein fractionations have also been attained with agar as a stabilizer. The earliest reports appear to be those of Field, Teague and Buxton (64–66), in 1907, who made an investigation of such substances as antibodies, antigens, hemolysins, agglutinins, toxins and antitoxins. Modern papers dealing with the use of agar as a stabilizing agent for the ionographic fractionation of proteins include those of Davis *et al.* (67) and Gordon *et al.* (68).

LOCALIZATION AND QUANTITATIVE ANALYSIS

To determine the location of the various zones on an ionogram occupied by the individual components of a mixture, a number of procedures have been developed. They include the following: the dye-elution method; direct photometric scanning by transmitted or reflected light; the use of fluorescence or absorption phenomena with respect to ultraviolet light; marking with radiotracers or, in some cases, dyes; direct nitrogen or amino acid analysis of proteins, in which the staining procedure is eliminated and the protein is determined by micro-Kjeldahl, polarography or some common protein test (1).

AREAS OF CONTROVERSY

Because of the very rapid growth of ionography, the widely varied background of the investigators who have adopted the technique and

the too-often apparent lack of understanding of the fundamental principles involved, it was inevitable that a number of controversial issues should have arisen. Some of these will now be considered.

Design of Apparatus. While each of the principal designs of apparatus has been successfully applied in simple ionographic fractionations, some modifications possess inherent advantages when mobility determinations are to be made. Thus the ridgepole technique and the vertical technique are less readily applicable to the determination of mobilities than the other principal designs.

Separation of Electrode Products From the Buffer Solution. An adequate separation of the electrode products from the buffer vessels ought now to be a part of standard practice, since anomalous results can so often be traced to this source. This separation may be accomplished by interposing simple agar-KCl salt bridges between the buffer vessels and the electrode vessels (11, 12). In some cases the use of reversible electrodes may be advisable. Silver-silver chloride electrodes immersed in a 2.5 molar potassium chloride solution and separated from the buffer vessels by the agar-KCl salt bridges are satisfactory for this purpose.

Control of Evaporation. Although evaporation of solvent has actually been promoted in some instances as an aid in the empirical fractionation of complex mixtures (21, 22), it is more customary to attempt to reduce it to a minimum in order to minimize nonreproducible chromatographic effects. In the sandwich technique and solvent immersion techniques, the factor of evaporation of solvent is not difficult to deal with, although certain other limitations enter in to limit their general application. In any of the open-strip methods, the vapor-space can be held to a minimum, lowered temperatures may be used, and a combination of buffers of low ionic strength with an increased potential gradient may be resorted to. Where evaporation must be prevented altogether, glycerol, propylene glycol or similar substances may be added to the buffer solution to lower its vapor pressure below that of the water in equilibrium with the same vapor space (43, 69). Under these circumstances no net evaporation of water from the paper strip occurs and mobilities of migrants remain constant for many hours.

Equilibration Time and Constant Water-to-Paper Ratio. The electromigration velocity of a migrant decreases practically to zero as the water-to-paper weight ratio (or more strictly, the weight ratio of buffer solution to paper) approaches zero, and increases in magnitude up to the velocity in free-solution electrophoresis in the limit, as the water-to-paper ratio increases. Too often, insufficient time is allowed for equilibrium conditions to be reached, i.e. for the attainment of a constant water-to-paper ratio, before applying the migrant. This situation may arise whatever the design of the apparatus. In the sandwich technique, the water-to-paper ratio is affected by an additional variable, namely, the pressure with which the plates are pressed together. If simple spring clamps are used for this purpose, the ratio of buffer solution to paper may vary from point to point throughout the length and breadth of the paper filler. Under such circumstances, mobility measurements are inaccurate and it may be difficult to duplicate the results of an experiment even though the same type of apparatus is used.

Comparison of Schlieren and Optical Density Diagrams. The patterns obtained by means of the Schlieren method when used in conjunction with the moving-boundary apparatus, and those derived from transmission densitometer readings on ionograms, differ not only in degree but in kind (1, 44). When optical density is plotted against the distance of migration of the components of a colored mixture along an ionogram, a plot is obtained wherein the area under an individual peak is proportional to the amount of protein component under that peak. In the Schlieren pattern, on the other hand, when the change of concentration, C with respect to distance, (i.e. dC/dX), is plotted against the distance, x, the area under the curve is proportional to the concentration of the material present. Thus, although the curves from a complex mixture such as blood plasma proteins may show a certain similarity in shape, no real correspondence is to be expected because of the intrinsic, fundamental difference in the nature of the two somewhat similar patterns.

Area Under Peaks; Dropping Perpendiculars Versus Extending Curves to Baseline. A difference of opinion among investigators in this field exists as to whether, in the determination of areas under the individual peaks of a curve (optical density versus distance of migration along the ionogram), perpendiculars should be drawn downwards from the midpoints of the valleys or whether the curves should be extended, as shown in figure 2. That there is a difference between the two results cannot be argued. In the particular

case shown here, namely the alpha-2 globulin fraction of human plasma proteins, the difference amounts to 7.5%. If the goal is to obtain simple 'ratios' between various constituents, then either treatment would seem satisfactory. However, in those instances where the actual amounts of individual components are desired, it will readily become apparent on a little reflection that the drawing of perpendiculars from the valleys to the base-line can yield the correct result only when all the curves are of the same shape and size. Needless to say, integrators which incorporate this principle in the determnation of areas suffer from the same limitations.

Constant Current Versus Constant Potential. The correct expression for field strength for a system containing buffer and paper fiber is V/l or $i/q_a \cdot K_p$, which are equivalent. In these expressions the meanings of the symbols are as follows: V is the voltage applied across the length of paper, l and i is the current flowing through the paper with an effectual cross-sectional area of q_a, saturated with a buffer, and the specific conductivity of the paper-buffer system is K_p. Since it is easier to measure the voltage drop across the strip, and the length of the paper strip from buffer surface to buffer surface, V/l is the most convenient expression to use for field strength. Even though $i/q_a \cdot K_p$ is equivalent to V/l, the former expression is not readily handled because of the difficulty involved in evaluating the quantity, q_a, that is, the effective cross-sectional area (1).

In simple empirical fractionation experiments, it really doesn't matter very much whether current or potential or both are held constant, if the range of variation is not too large. However, since 'mobility' represents the linear distance a particle moves in unit time per unit field strength, it is evident that in experiments involving the determination of 'mobilities,' the potential gradient must be kept constant so that the conditions for the definition of mobility may be fulfilled. It is hardly reasonable to hold current constant and let the potential gradient vary when calculating mobilities. It can only be held constant when it causes the potential gradient to be constant, that is, when the specific conductance does not change.

The potential gradient can, of course, be measured directly only when the conductor is of uniform corss-sectional area and hence of uniform resistance, a condition which can be attained in the proper design of ionographic experiments. If the specific conductance is not constant and changes throughout the various regions of the strip, the true potential gradient cannot be obtained by dividing the potential across the strip by its length, and the 'mobility' of a migrant cannot be calculated under these circumstances.

Relative Uptake of Dye and Purity of Dyes. It was shown rather early in the development of ionography that the distribution of dye between the separated protein fractions of blood was not necessarily identical with the proportions of the various proteins as determined by other methods. Discrepancies can arise due to differences in the relative rate of uptake of dye as well as to differences in the relative equilibrium amounts. Since it cannot be assumed that the uptake of dye bears a similar or simple relationship to the concentration of various protein fractions, it is necessary to resort to careful calibration procedures, based, for example, on nitrogen determinations, if intensity of color is to be used as an accurate guide to the absolute amounts of protein fractions on an ionogram. With reasonable care the inadequacies of present staining techniques can be reduced to a level where the applicability of ionography to the majority of problems met in the clinical chemical laboratory is not restricted to any appreciable extent.

The variability in purity of similar dyes between various batches from the same source, and between lots from different sources, is also a factor which has led to much confusion in the literature. Despite a claim made by an author for good staining ability and contrast for a particular dye, workers in another laboratory have not always been able to achieve satisfactory results, due, apparently, in many cases to the fact that the dyes were purchased from different sources. The chromatographic analyses of lipid stains, as discussed in a later part of this paper, has revealed the fact that they generally contain many components. In the case of Sudan black B, for example, the dye contained several differently colored substances, only one of which appeared to stain lipids.

Electroosmosis. It has been pointed out many times that in electromigration in stabilized media, the mobility of positively charged ions might be expected to be increased while that of negatively charged ions might be decreased, due to the phenomenon of electroosmosis. In the experiments where mobility values are a concern, the problem must be dealt with. It has been handled in two principal ways, namely, attempts have been made to reduce the effects of electroosmosis to the status of a negligible factor, or attempts

have been made to measure the effect and to apply suitable correction factors. The attempts at reduction include the interposing of cellophane sheet barriers between the ends of the paper strips and that part of the strips which dip into the buffer solution. In this way the region of the strips through which electromigration is taking place is isolated from the effects of water shifts due to electroosmosis, while at the same time electrical conductivity is essentially unimpaired (70). Other investigators have found that if the electrodes are attached directly to the moist paper strips, in the sandwich technique, electroosmosis is drastically reduced (71). Attempts have also been made to alter the charge on the cellulose fibers of the paper (72–75).

The attempts at measuring electroosmosis are generally based on the use of easily detectable substances which exhibit a negligible native electromigration tendency in free solution and which are not absorbed on the surfaces of the stabilizing medium. The movement of such substances has been taken as a measure of the extent of electroosmosis. This solution of the problem suffers from the fact that due to the screening effect of the stabilizer, the molecular shapes and volumes of the indicator and migrant materials should be closely alike if the so-called correction factors are to be meaningful (1, 44).

Mobility Relationships. Some of the more fundamental aspects of ionography, as far as mobilities are concerned, include the relationships that exist between the movement of the migrant and such factors as time, potential gradient, pH of the buffer solution and its ionic strength, temperature, wetness, adsorption, nature of the paper and of the migrant and the role of electroosmosis (1, 44). The mobility of a migrant is also influenced by such factors as the method of supporting and wetting the paper strips, the thoroughness with which the electrode products are separated from the paper itself, capillary siphoning of liquid through the strip, the extent of evaporation of solvent from the paper, the procedure adopted in drying the strip after a run, the point of application of the migrant to the strip, and the relative amounts of water held by various types of paper (1).

Mobility values determined in stabilized media are, under the usual experimental conditions, lower than those observed under comparable conditions in free solution, although it is possible to reduce the difference between the two sets of values to a negligible factor. If the 'mobility' of a migrant is plotted against 'time' and an extrapolation of the mobility to 'zero time' is made, it has been found, with bovine serum albumin, beta lactoglobulin and egg albumin as migrants, that the agreement with 'free solution values' is within experimental error. So-called 'free-solution mobilities' for the same substances and also for bovine gamma globulin have been obtained by direct experiment (1). This was accomplished by working at low wattage values as regards the energy expenditure in the strips, with short strips (20 cm), at a temperature of 4°C, and at maximum wetness values for the paper.

A number of authors have described the development of conversion factors relating mobilities determined in stabilized and in non-stabilized media. These include Kunkel and Tiselius (3) Woods and Gillespie (76), and McDonald and co-workers (1). Although differing in the assumptions made as to the factors responsible for the divergence in the raw mobility values obtained in stabilized and in non-stabilized media, the three different conversion factors suggested by these authors all appear to yield good agreement with free solution mobilities when applied to mobilities determined in stabilized media. This fact can be interpreted to mean that much is yet to be learned regarding electromigration in general.

Photometric Scanning and Applicability of Beer's Law. Numerous ingenious arrangements for scanning paper ionograms have been described (1). The principal types would include *a*) transmission scanners, often coupled with transparentization of the ionograms with mixtures of paraffin oils plus additives such as alpha bromonaphthalene; *b*) reflectance scanners involving the measurement of light reflected from the surface of the dry ionograms; *c*) ultraviolet absorption techniques, where the materials comprising the zones on the ionograms absorb in this region of the spectrum or are marked with ultraviolet-absorbing substances; *d*) infra-red absorption techniques (77). The literature covering the application of Beer's law to the scanning of ionograms is quite controversial (1, 23), reflecting a need for continued careful experimental work in this area.

CONTEMPORARY ADVANCES

Very few scientific fields have developed as rapidly as that of ionography. As the quantitative character of the technique continues to be improved upon, the areas of controversy are being narrowed, and new developments that extend the applications of the technique and, increase its

range are constantly being reported. Three such developments from the authors' laboratory are reported below.

A New Dying Procedure (Pre-staining) for Lipoproteins. During very recent years there has been an upsurge of interest shown in the study of lipoproteins, largely because of their implication in the genesis of atherosclerosis. One of the most convenient ways of separating the alpha and beta lipoproteins from blood is to fractionate serum ionographically. In previous studies of lipids and lipoproteins by this method, in paper-stabilized electrolytes, the chief difficulties encountered were in the inadequate staining of the lipoprotein bands, in the retention of the stain by the paper itself, and in the undue length of time often required for the staining process—sometimes a matter of several hours. By the new procedure described here, involving pre-staining of the lipids before application of the serum sample to the paper, these difficulties have been largely overcome.

Stains which have been used for the detection of lipoproteins on ionograms include: Sudan black B (78, 79), acetylated Sudan black B (80, 81), Sudan III (82, 83), Sudan IV (84), oil red O (85), oil black (86), and combined Ciba blue (BZL)-Sudan black (87).

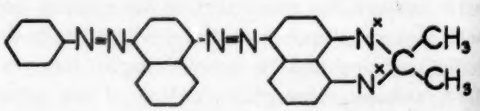
The most favorable experience in this laboratory with the conventional method of staining the ionograms (namely, after a run) was obtained with Sudan black B (78, 79) or so-called acetylated Sudan black B (80, 81), which produce ionograms having dark blue bands corresponding to the areas covered by lipoproteins, against a light blue background.

When preliminary experiments with paper chromatography using various solvent systems indicated that commercial Sudan black B (Hartman-Leddon Co., NZb-5) was a multicomponent mixture, an attempt was made to isolate the staining component in order to obtain an improved stain. Using column chromatography with a celite-silicic acid packing, and petroleum ether (b.p. 30–60°C) as solvent, the column was washed down with the solvent for 40 hours at room temperature, under the stimulus of reduced pressure at the lower receiving end. Four major components were collected. On testing the ability of the various components to stain lipoproteins, it was found that this property was associated with only one of the fractions. When an absorption spectrum was run on this component, the region of maximum absorption was found to be 595–605 millimicrons. This purified fraction of Sudan black B still gave a colored background to the ionograms when used in the conventional way.

Casselman has reported the use of acetylated Sudan black B as a stain for lipids on paper chromatograms (80). The procedure for the preparation of the acetylated Sudan black B is described by Lillie and Burtner (81), who used it as a histochemical reagent. Briefly, the procedure is as follows: Two grams of Sudan black B are added to 60 milliliters of pyridine and 40 milliliters of acetic anhydride, and the mixture is allowed to stand overnight. The dye is now precipitated from 3 liters of water, dried and dissolved in acetone.

In using the acetylated Sudan black B to develop ionograms with lipoprotein zones, the following staining procedure was employed. A saturated solution of the dye in a 70% ethanol-water mixture was heated to boiling, then allowed to cool before repeated filtration, first through No. 1 Whatman filter paper and subsequently through No. 42 Whatman. The paper strips were immersed in the staining solution at 40°C for a period of 20 minutes, then washed several times in 50% ethanol-water solutions. The acetylated dye produced a deeper color in the lipoprotein zones of the ionograms and the background coloration was lightened, but the result still left much to be desired.

The actual mechanism of the acetylation procedure is unknown. The structural formula for Sudan black B is given by Conn (88) as:



Conn reports the corresponding empirical formula as $C_{29}H_{22}N_6$ (although if the starred nitrogen atoms are to be trivalent, $C_{29}H_{24}N_6$ would seem to agree with the structural formula he gives), and the molecular weight given (554.414) does not agree with either the structural or empirical formula. The acetylation could possibly take place at the positions indicated (*) on the structural formula, but another possibility which cannot be overlooked is that the acetylation is simply responsible for the removal of an impurity in the dye and does not actually combine with the Sudan black B molecule itself to alter its structure. This latter view would seem to be supported by the following observations, although any final decision on the matter must await a more thorough investigation. Preliminary column chromatographic studies indicate

that the same major fractions are obtained with acetylated Sudan black B as with Sudan black B itself, and a comparison of the absorption spectra of the fractions did not reveal any significant difference between the regular and acetylated dyes.

It was at this point that McDonald and Bermes investigated the feasibility of prestaining the lipid and lipoportein fractions in the serum before subjecting an aliquot of the sample to ionographic fractionation (89). After a number of preliminary experiments, the following procedure was worked out and was found to yield good results.

By the new procedure, the lipoproteins are prestained directly in the blood serum sample before application to the paper, as follows: To 1 milliliter of blood serum in a small test tube there is added slowly (to avoid precipitation) 0.1 milliliter saturated solution of acetylated Sudan Black B in 95% ethanol (or for smaller relative amounts, the same 1–10 ratio of dye to serum). The tube containing the serum is now shaken and allowed to stand at room temperature for 30 minutes (although even 3–5 minutes will yield fair results). The excess alcohol is evaporated by applying a suction to the tube and allowing a fine stream of nitrogen (or air) to be pulled over the serum through a thinly-drawn capillary tube. Some of the dye may settle to the bottom of the tube. This may be removed by centriguging the sample and drawing off the liquid.

Five microliters of the serum containing the stained lipoproteins is now applied from a micropipet to the filter paper (Whatman No. 1, 0.5 inch width) as a streak across the paper ribbon. The horizontal open-strip method, employing the Precision Ionograph[1], was used in making the separations. The other experimental conditions generally used for optimal results were: veronal buffer; pH, 8.6; ionic strength 0.05; potential gradient 6 v/cm; atmosphere, water-saturated helium at 0–5°C, although runs in a simple water-saturated air atmosphere at room temperature were also quite satisfactory. An adequate separation is obtained in 4–5 hours. Both the alpha and the beta lipoproteins migrate in the usual manner and appear as blue zones against a white background.

Since the dye is apparently physically dissolved in the lipoproteins and is insoluble in water, there is no smearing of the colored zones. On investigating the ionographic behavior of Sudan black B and acetylated Sudan black B in acetate and veronal buffer systems (40–80% propanol) it was observed that neither of the dyes showed any movement under the influence of an electric field. No dissociation or breakup of the colored materials was evident, as is observed when serum proteins are prestained with bromphenol blue (90). This can be explained as follows: the Sudan black B apparently dissolves physically in the lipid-protein moiety, whereas the protein-bromophenol blue complex involves an ionic bond and an equilibrium which can be upset due to the leaking away of the bromophenol blue ions from the complex, under the influence of the electric field. Using the same technique except that the Sudan black B was replaced by Sudan III, Sudan IV and oil red O, the results were much less satisfactory.

The new technique eliminates a long and tedious staining procedure including washing of the ionogram, conserves rather costly reagents, and yields crisp, well-demarked zones which enable the relative amounts of alpha and beta-lipoproteins to be determined more accurately than was formerly the case.

Interaction Studies. In recent years as more biological processes have begun to be viewed on a molecular level, the significance of protein interaction studies has been extended. The results have aided in the elucidation of many biological functions of the proteins, including their action as transporting agents in the circulatory system, their role in enzymatic catalysis, and their effect on drug activity.

There are, however, more immediate chemical and mechanistic consequences to be gathered from the study of the protein complex. Klotz (91) has enumerated the important purposes of interaction studies, as follows: *1*) A determination of the number of interacting molecules that are held by a given protein molecule, under specified environmental conditions. *2*) A determination of the maximum number of sites on a protein molecule available to a given interacting species. *3*) A thermodynamic consideration of the strength of the bond between the protein and any given interacting species. *4*) A quantitative statement as to the effect of the environment on the energy of combination. *5*) Quantitative information on the structural characteristics of the interacting molecules which favor combination with the protein. *6*) A quantitative elucidation of the molecular and configurational nature of the site on the protein at which a given species is bound.

The value of moving-boundary procedures as

[1] Precision Scientific Co., Chicago 47, Ill.

applied to protein-interaction studies has been demonstrated by Longsworth and MacInnes, (92), by Alberty and Marvin (93) by Smith and Briggs (94) and by Yang and Foster (95). Attempts at evaluation of the binding constants have been successful mainly in the case in which the rate of dissociation of the complexes is greater than the rate of electrophoretic resolution.

Interaction studies using the ionographic technique have been described by Spitzer and McDonald, who have been able to obtain qualitative evidence of interaction using filter paper as the stabilizer (90). Equilibrium dialysis experiments were carried out using bovine serum albumin (BSA) and bromphenol blue (BPB) in both a phosphate buffer (0.05M, pH 7.4, 4°C), and a veronal buffer (0.05M, pH 8.6, 4°C). Similar experiments were conducted using polyvinylpyrrolidone (PVP) and bromphenol blue. The results of this work were applied to calibrate the ionographic method. Two ionographic procedures were employed to study these systems. In the first method, 2–15 lambdas of the migrants, either the BSA-BPB or PVP-BPB systems, were used. After the passage of current for a period of time long enough to separate some of the faster moving BPB but short enough to retain some of the equilibrium mixture, the strips were dried and subjected to a direct spectrophotometric analysis (585 mμ). An asymmetric pattern was found, representing separated bromphenol blue and the bromphenol blue in BSA-BPB or PVP-BPB equilibrium mixtures. Two blue zones of slightly different hue could be distinguished visually on the filter paper. It was observed that on prolonged passage of the current, the BPB could be separated completely from the albumin. This observation is of interest since many workers have used BPB in a mistaken attempt to 'label' the albumin of serum and to follow its migration during the course of the experiment.

Another ionographic procedure employed to demonstrate the formation of complexes involved the calculation of the 'constituent mobility' of a given compound. BSA, PVP or BPB complexes of these compounds were employed as migrants in BPB-buffer solutions. Electromigration of PVP or BSA in this medium ought to be characteristic of their complexes. Should their mobilities be different from those in non-BPB buffers, an indication of complexing is evident. Mobility measurements were obtained in phosphate buffer (pH 7.4, 0.05M) and in phosphate buffer (pH 7.4, 0.05M, 1.785×10^{-3}M with respect to BPB).

It appeared evident that the BPB complexes of PVP and BSA have a greater net negative charge than the non-complexed species. That is, the mechanism of binding of BPB to either PVP or BSA is such that an increased negative charge is added to the colloidal species. PVP had its direction of movement reversed when the dye was introduced into the buffer solutions, thus moving to the anode. It was interesting to note the appearance of a white band that moved with a mobility greater than PVP, BSA, and BPB. This zone had a similar mobility regardless of the large molecular species that was used.

Roth and Kallee (96, 97) have reported somewhat similar interaction experiments. Amido black 10B, azorubin S, or naphthol yellow were added to the buffer solution and the mixture was used to moisten the filter paper strips. Albumin was placed on the strips and allowed to migrate. A white zone on the strip was found in front of or behind the albumin, depending on the mobility of the dye. In the interpretation of these occurrences it was suggested that the phenomenon might be dependent on the formation of albumin-dye complexes.

Ionographic Isoelectric Separations. In a recent series of papers (98–100) Kolin has described the separation and concentration of a number of substances in a pH-gradient field combined with an electric field to yield what he has termed 'isoelectric spectra.' The principle involved is not unlike that used by Williams and Waterman (101, 102) except that the pH gradient in Kolin's U-shaped channel was continuous and semimicro in scale while that of Williams and Waterman was a step-wise proposition involving 14 compartments filled with ampholytes in solutions of different pH separated by parchment membranes. After a 60-hour run in the latter arrangement the concentration was found to be greatest in compartments having a pH nearest the isoelectric pH of the ampholyte. Kolin has achieved separations of hemoglobin, cytochrome *c*, catalase, etc. in a matter of minutes.

The apparatus used by Kolin consists of the U-shaped cell of square cross section made from polymethacrylate. An acid buffer containing enough sucrose to make it heavier than any other liquids in the cell fills the bottom half of the U. Basic buffer fills the top parts of the two U-tube arms and the electrode wells which connect with the arms at the top. The density of the suspension being investigated is adjusted to a point intermediate between that of the acidic

and basic buffer and is introduced between them by means of a long thin-nosed pipet, and the potential is then applied across the electrodes.

In the authors' laboratory, the isoelectric technique in electrophoretic analysis has been modified and adapted to ionographic separations in both paper- and starch-stabilized media (103). How this is accomplished using paper-stabilized media can be explained by reference to figure 1. The frame in the apparatus is loaded with two paper strips separated by a spacing of about 4 centimeters. One end portion of the strip which dips down into the buffer vessel is torn off each of the two strips, leaving only one end of each strip, at opposite ends of the frame, to make contact with the liquid in the buffer vessels. In one buffer vessel, a solution 1 molar with respect to both HCl and NaCl is placed, while in the other, a solution 1 molar in NaOH and NaCl is poured. The paper strip in the acid solution is also wetted with the acid, and the strip in the basic solution, with the base. At right angles to the length of the strips in the frame, a short length of filter paper wetted simply with distilled water is placed so as to connect the longer paper strips electrically, in the form of a bridge, and the migrant is applied as a streak across the paper bridge at its midpoint. The potential is then applied. Since the main strips in the frame, being wetted with a strongly acidic or basic solution, are excellent electrical conductors, the larger portion, 90% or so, of the potential drop occurs across the narrow paper bridge. With 450 volts applied across the frame, a voltage drop of about 400 volts occurs across the paper bridge, which may be 4 centimeters in length. Since the current is very low, evaporation is not a problem, and under the impetus of 100 v/cm, a migrant such as bromophenol blue moves very rapidly toward the positive pole—in this case the acid solution. At first the initial narrow zone widens out, but the front of the migrating zone soon reaches a point beyond which it ceases to move and the tail of the zone moves up, resulting in a clean-cut line of high color intensity. The whole process under the conditions described requires from 1.5 to 2 minutes.

Amino acid mixtures as well as plasma proteins have been fractionated, under the conditions described above, and also with the use of citric acid and sodium citrate as the acidic and basic buffer solutions, respectively. While the results obtained with amino acid mixtures and other lower molecular weight materials such as bromophenol blue are satisfactory, the fractionations of plasma proteins so far obtained are not nearly as clean-cut as obtained with the more traditional ionographic procedure.

Future of the Technique. It was probably inevitable, although unfortunate, that too large a fraction of the approximately 1000 papers which have been published on the technique of ionography report on investigations which were carried out without sufficient appreciation of, or attention to, the fundamental principles underlying the technique. Because of this situation, a comparison of the results from different laboratories is often invalid, due to the unknown extent of the deviations in experimental details in the different laboratories. Anomalous results which arise from errors in technique rather than the properties of the migrants can often be traced to the same cause. This chaotic situation is, fortunately, now being ironed out, as more and more workers become cognizant of the responsibility to reassess their experimental procedures and to incorporate the essential features which will make their reports meaningful.

For clinical chemical problems where the material is limited, multiple analyses are required, and rapidity of analysis is of importance, ionography would seem made to order. On consideration of the rate at which the technique is currently being adopted in various clinical chemical laboratories, and of the greater heed being paid to the fundamental principles involved, it can be said that the potential range of usefulness of ionography in the routine laboratory and in research is such as to place it in the very forefront of clinical laboratory procedures.

REFERENCES

1. McDonald, H. J., R. J. Lappe, E. P. Marbach, R. H. Spitzer and M. C. Urbin. *Ionography; Electrophoresis in Stabilized Media.* Chicago: Year Book Publ., 1955.
2. Cremer, H. and A. Tiselius. *Biochem. Ztschr.* 320: 273, 1950.
3. Kunkel, H. G. and A. Tiselius. *J. Gen. Physiol.* 35: 89, 1951.
4. von Klobusitzky, D. and P. König. *Arch. exper. Path. u. Pharmakol.* 192: 271, 1939.
5. Consden, R. and W. M. Stanier. *Nature* 170: 1069, 1952.
6. Markham, R. and J. D. Smith. *Nature* 168: 406, 1951.
7. Berraz, G. *Anal. Asoc. quim. argent.* 31: 96, 1943.
8. Berraz, G. *Anal. inst. invest. cient. tecnol., Univ. nac. Litoral* 12–13: 67, 1943.
9. Michl, H. *Monatsh. Chem.* 82: 489, 1951.

10. Grassmann, W. and K. Hannig. *Naturwissenschaften* 37: 496, 1950.
11. McDonald, H. J., M. C. Urbin and M. B. Williamson. *Science* 112: 227, 1950.
12. McDonald, H. J., M. C. Urbin and M. B. Williamson. *J. Colloid. Sci.* 6: 236, 1951.
13. Durrum, E. L. *J. Am. Chem. Soc.* 72: 2943, 1950.
14. Flynn, F. V. and P. de Mayo. *Lancet* 261: 235, 1951.
15. Robbins, J. and J. E. Rall. *Proc. Soc. Exper. Biol & Med.* 81: 530, 1952.
16. Brattsten, I. and I. Nilsson. *Ark. kimi* 3: 337, 1951.
17. Durrum, E. L. *J. Am. Chem. Soc.* 73: 4875, 1951.
18. Grassmann, W. In: *The Chemical Structure of Proteins*, edited by E. W. Wolstenholme, and M. P. Cameron. Boston: Little, 1954, p. 195.
19. Haugaard, G. and T. D. Kroner. *J. Am. Chem. Soc.* 70: 21, 1948.
20. Strain, H. H. and J. C. Sullivan. *Anal. Chem.* 23: 816, 1951.
21. Macheboeuf, M., P. Rebeyrotte, J. M. Dubert and M. Brunerie. *Bull. Soc. chim. biol.* 35: 334, 1953.
22. Macheboeuf, M., J. M. Dubert and P. Rebeyrotte. *Bull. Soc. chim. biol.* 35: 346, 1953.
23. Block, R. J., E. L. Durrum and G. Zweig. *Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press, 1955.
24. Kunkel, H. In: *Methods of Biochemical Analysis*, edited by D. Glick. New York: Interscience, 1954, p. 141.

24a. Lederer. *Introduction to Paper Electrophoresis*. New York: Elsevier, 1955.

25. Tiselius, A. and P. Flodin. In *Advances in Protein Chemistry*, edited by M. L. Anson and J. T. Edsall. New York: Academic Press, 1953, p. 461.
26. Wunderly, C. *Die Papierelektrophorese; Methoden und Ergebnisse*. Aarau, Switzerland, Sauerländer, 1954.

26a. Alberty, R. A. In: *The Proteins*, edited by H. Neurath and K. Bailey. New York: Acad. Press, 1953, p. 461.

27. Trautman, R. Abstr. Am. Chem. Soc. Mtg., Chicago, Sept., 1953, p. 39C.
28. Graf, E. and P. H. List. *Naturwissenschaften* 40: 273, 1953.
29. Clark, W. M. In: *Topics in Physical Chemistry*, 2nd ed. Baltimore: Williams & Wilkins, Baltimore, Md., 1952, p. 139.
30. Holmes, H. N. In: *Medical Physics*, edited by O. Glasser. Chicago: Year Book Publ., 1944, p. 257.
31. Stauffer, R. E. In: *Technique of Organic Chemistry*, edited by A. Weissberger. New York: Interscience, 1950, p. 313.
32. Slater, R. J. and H. G. Kunkel. *J. Lab. & Clin. Med.* 41: 619, 1953.
33. Schneider, G. and G. Wallenius. *J. Clin. & Lab. Invest.* 3: 145, 1951.
34. Enriquez, de Salamanca, F., C. Zuazo de Leon, J. Gonzalez Alvarez and Otero de la Gandara. *J. Arch. med. exper.* (Madrid) 16: 359, 1953.
35. Wiedermann, D. *Lek. Listy* 8: 468, 1953.
36. Cumings, J. N. *J. Neurol., Neurosurg. & Psychiat.* 16: 152, 1953.
37. Bauer, H. *Deut. Ztschr. Nervenh.* 170: 381, 1953.
38. Bücher, T., D. Matzelt and D. Pette. *Klin. Wchnschr.* 30: 325, 1952.
39. Peters, H. J. *Chem. Weekblad* 49: 248, 1953.
40. Esser, H. and F. Heinzler. *Klin. Wchnschr.* 30: 600, 1952.
41. Goa, J. *J. Clin. Lab. Invest.* 3: 236, 1951.
42. Adjutantis, G. *Nature* 173: 539, 1954.
43. McDonald, H. J. *J. Chem. Educ.* 29: 428, 1952.
44. McDonald, H. J., R. J. Lappe, E. P. Marbach, R. H. Spitzer and M. C. Urbin. *Clin. Chemist* 5: 35, 1953.
45. Boyd, W. C. *J. Am. Chem. Soc.* 67: 1035, 1945.
46. Gomori, G. *Proc. Soc. Exper. Biol. & Med.* 62: 33, 1946.
47. Miller, G. and R. Golder. *Arch. Biochem.* 29: 420, 1950.
48. Hawk, P., B. Oser and W. Summerson. *Practical Physiological Chemistry*, 13th ed. New York: Blakiston, 1954.
49. Koch, F. and M. Hanke. *Practical Methods in Biochemistry*. Baltimore: Williams & Wilkins, 1953.
50. Perkin-Elmer Corporation, Instruction Manual—Portable Tiselius Electrophoresis Apparatus Model 38. Norwalk, Conn., 1951.
51. Michaelis, L. *Biochem. Ztschr.* 234: 139, 1931.
52. Albright, E. C., W. P. Deiss and F. C. Larson. *Proc. Cent. Soc. Clin. Res.* 26: 7, 1953.
53. Flodin, P. and J. Porath. *Biochim. et biophys. Acta* 13: 175, 1954.
54. Fønss-Bech, P. and C. H. Li. *J. Biol. Chem.* 207: 175, 1954.
55. Kunkel, H. G. Abstr., Am. Chem. Soc. mtg., Chicago, Sept., 1953, p. 43C.
56. Kunkel, H. G. and R. J. Slater. *Proc. Soc. Exper. Biol. & Med.* 80: 42, 1952.
57. Larson, F. C., W. P. Deiss and E. C. Albright. *J. Clin. Investigation* 33: 230, 1954.
58. Nikkila, E. A., E. Haahti and R. Pesola. *Acta chem. scandinav.* 7: 1222, 1953.
59. Raacke, I. D. and C. H. Li. *Biochim. et biophys. Acta* 14: 290, 1954.
60. Siliprandi, D. and N. Siliprandi. *Biochim. et biophys. Acta* 14: 52, 1954.
61. Siliprandi, N., D. Siliprandi and H. Lis. *Biochim. et biophys. Acta* 14: 212, 1954.
62. Smithies, O. *Nature* 175: 307, 1955.
63. Wetter, L. R. *Canad. J. Biochem. and Phys.* 32: 20, 1954.
64. Field, C. W. and O. Teague. *J. Exper. Med.* 9: 86, 1907.
65. Field, C. W. and O. Teague. *J. Exper. Med.* 9: 222, 1907.

66. TEAGUE, O. AND B. BUXTON. *J. Exper. Med.* 9: 254, 1907.
67. DAVIS, W. W., T. V. PARKE AND B. GAMEWELL. Abstr., 12th Internat. Cong. Pure and Appl. Chem., New York, Sept., 1951, p. 65.
68. GORDON, A. H., B. KEIL AND K. SEBESTA. *Nature* 164: 498, 1949.
69. MCDONALD, H. J. *Science* 121: 403, 1955.
70. MICHL, H. *Monatsh. Chem.* 83: 737, 1952.
71. WOOD, S. E. AND H. H. STRAIN. *Anal. Chem.* 26: 1869, 1954.
72. HAUSER, E. A. AND J. E. LYNN. *Experiments in Colloid Chemistry.* New York: McGraw-Hill, 1940, p. 42.
73. JERMYN, M. A. AND R. THOMAS. *Nature* 172: 728, 1953.
74. KALLEE, E. *Ztschr. Naturforsch.* 7*B*: 661, 1952.
75. RUTTER, L. *Nature* 166: 273, 1950.
76. WOODS, E. F. AND J. M. GILLESPIE. *Austral. J. Biol. Sc.* 6: 130, 1953.
77. GOULDEN, J. D. S. *Nature* 173: 646, 1954.
78. SWAHN, B. *Scand. J. Clin. Lab. Invest.* 4: 98, 1952.
79. SWAHN, B. *Scand. J. Clin. Lab. Invest.* 5 5 (suppl. 9): 1, 1953.
80. CASSELMAN, W. G. B. *Biochim. et Biophys. Acta* 14: 450, 1954.
81. LILLIE, R. D. AND H. J. BURTNER. *J. Histochem.* 1: 8, 1953.
82. FASOLI, A. *Lancet* 262: 106, 1952.
83. FASOLI, A. *Acta med. scandinav.* 145: 233, 1953.
84. ROSENBERG, I. N. *Proc. Soc. Exper. Biol. & Med.* 80: 751, 1952.
85. DURRUM, E. L., M. H. PAUL AND E. R. B. SMITH. *Science* 116: 428, 1952.
86. BAUDOUIN, A., J. LEWIN AND P. HILLION. *Compt. rend. Soc. biol.* 147: 1036, 1953.
87. LOEFFLER, W. AND C. WUNDERLY. *J. Clin. Path.* 6: 282, 1953.
88. CONN, H. J. *Biological Stains*, 6th ed.; Geneva, N. Y. C., Biotech Publ., 1953.
89. MCDONALD, H. J. AND E. W. BERMES. *Biochim. et Biophys. Acta* 17: 290, 1955.
90. SPITZER, R. H. AND H. J. MCDONALD. *Federation Proc.* 14: 285, 1955.
91. KLOTZ, I. M. *Cold Spring Harbor Symp. Quant. Biol.*, XIV: 97, 1949.
92. LONGSWORTH. L. G. AND D. A. MACINNES. *J. Gen. Physiol.* 25: 507, 1942.
93. ALBERTY, R. A. AND H. H. MARVIN. *J. Phys. and Colloid Chem.* 54: 47, 1950.
94. SMITH, R. F. AND D. R. BRIGGS. *J. Phys. & Colloid Chem.* 54: 33, 1950.
95. YANG, J. T. AND J. F. FOSTER. *J. Am. Chem. Soc.* 75: 5560, 1953.
96. KALLEE, E. AND E. ROTH. *Ztschr. Naturforsch.* 8*B*: 34, 1953.
97. ROTH, E. AND E. KALLEE. *Ztschr. Naturforsch.* 8: 614, 1954.
98. KOLIN, A. *J. Chem. Phys.* 22: 1628, 1954.
99. KOLIN, A. *J. Chem. Phys.* 23: 407, 1955.
100. KOLIN, A. *Proc. Nat. Acad. Sc.* 41: 101, 1955.
101. SVENSSON, H. *Adv. in Protein Chem.* IV: 251, 1948.
102. WILLIAMS, R. R. AND R. E. WATERMAN. *Proc. Soc. Exper. Boil. & Med.* 27: 56, 1929.
103. MCDONALD, H. J. AND M. B. WILLIAMSON. *Naturwissenschaften.* In press.

OBSERVATIONS ON ALDOSTERONE IN HUMAN URINE[1]

JOHN A. LUETSCHER, JR. AND ROBERT H. CURTIS

From the Department of Medicine, Stanford University School of Medicine, San Francisco, California

THE STUDIES of Wintersteiner and Pfiffner (1), Kendall and Mason (2), and Cartland and Kuizenga (3) indicated that the amorphous fraction of adrenal cortical extract contained a hormone more active than any crystalline corticosteroid known at that time in maintaining life, growth, and electrolyte balance in adrenalectomized animals. Only very small amounts of this highly active mineralocorticoid were present in a mixture containing large amounts of closely related compounds. Methods, which were then used so successfully to isolate other corticosteroids, resulted in loss of much of the biologically active material. New methods of measurement and fractionation contributed greatly to recent progress in this field.

In 1947, Dorfman, Potts, and Feil (4) described a bioassay capable of detecting a few micrograms of desoxycorticosterone. In 1950, Deming, Johnson, and Luetscher, using a related bioassay, showed significantly increased sodium-retaining activity in urine extracts from edematous patients with nephrosis and heart failure (5). The high sodium-retaining activity fell when the patient improved and edema was eliminated (6). Hyman, working with adsorption columns, noted that the sodium-retaining corticoid was concentrated in a fraction more polar than desoxycorticosterone (7). In contrast with the considerable losses after adsorption on graphite or alumina, good recoveries were obtained after paper partition chromatography in systems as developed by Burton, Zaffaroni, Keutmann (8), and Bush (9).

In 1952, Tait, Simpson, and Grundy described similar studies on adrenal cortical extract (10, 11). Most of the mineralocorticoid activity was found in a fraction moving like cortisone on paper chromatograms in toluene and propylene glycol. Simpson and Tait (12) prepared several milligrams of purified material and described the unique properties of the new hormone, which was provisionally named electrocortin. In collaboration with Wettstein, Neher, v. Euw, Schindler and Reichstein (13), the substance was crystallized, was proved to be 18-oxo-corticosterone, and was given the name, aldosterone. The 11,18-hemiacetal appears to be the natural form (fig. 1).

Exchanges of information and material between the groups at Stanford, the Middlesex Hospital, and the Ciba Research Laboratories indicated that the sodium-retaining corticoid of urine behaved in all respects like aldosterone (14, 15). Both compounds showed the ultraviolet and infrared absorption spectrum and chemical reactions typical of a Δ^4-3-keto-steroid with the α-ketol side chain. They could not be separated in any of several chromatographic systems; their monoacetates and diacetates behaved similarly. Oxidation with periodate gave in each case a neutral steroid of similar properties.

From a concentrate of highly active, amorphous material from urine of a patient with nephrosis, Neher and Wettstein obtained a good yield of crystals identical in all respects with authentic aldosterone. The melting point was the same, and mixed melting point showed no depression. The infrared spectra of the free compound and of the diacetate were identical (16, 17). It seems very probable that the material of similar chromatographic and pharmacologic properties, found in the urine of normal men and various patients, is also aldosterone.

The great biological activity of aldosterone and its low concentration in body fluids have caused most workers to use a bioassay, in spite of its obvious deficiencies (18–22). At present, this is the only method capable of measuring aldosterone in urine of normal men (23). When larger amounts

[1] Work reported here was supported by a research grant (A-630) from the National Institute of Arthritis and Metabolic Diseases, and by a gift from the Smith, Kline, and French Foundation.

are present in urine, it is possible to use a two-step chromatographic separation with a physical or chemical method sensitive enough to detect the amount of aldosterone present (14, 24).

Doses of the order of 0.1 microgram of aldosterone significantly reduce sodium excretion in adrenalectomized rats under controlled conditions (18–22). Somewhat larger doses increase the excretion of potassium. There is little effect on water excretion at these levels (25, 26), but doses between 12 and 60 micrograms improve renal function and combat water intoxication in rats after adrenalectomy (27). Such large doses may also simulate some effects of cortisone or hydrocortisone (28), but no anti-inflammatory activity has been observed (29–31).

In man, Kekwick, Mach, Prunty, Thorn, and their co-workers (32–35) have shown that as little as 100 micrograms per day may be beneficial to patients with Addison's disease. Larger doses cause some sodium and water retention in normal man or in patients with rheumatoid arthritis (29, 35).

A small fraction of the aldosterone administered to two patients with adrenal insufficiency was recovered from the urine.[2] Urine was acidified to pH 1.0 and extracted with chloroform immediately and again after 24 hours. In urine of one adult patient, a total of 9.4 micrograms was found: this was less than 0.4% of the aldosterone (2.5 mg) given intramuscularly in aqueous solution during the 2-day period of urine collection. If the same rate of recovery of endogenous aldosterone is assumed to occur in extracts prepared in identical fashion from urine of patients with a functioning adrenal cortex, it would indicate a rate of secretion of aldosterone near 1 milligram per day in normal adults, increasing to 3–4 milligrams per day during sodium deprivation, and to more than 10 milligrams per day in severe congestive heart failure, hepatic cirrhosis, or nephrosis. Obviously, much more experience with such recovery experiments must be sought. When a child was given aldosterone in the same vehicle, site, and frequency, but in a much larger dose per unit of body weight (600 μg/2.7 kg in 2 days), a much higher rate of recovery (4.4%) was observed.

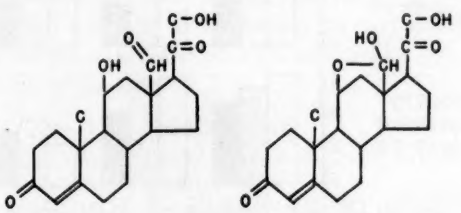
[2] The authors are indebted to Dr. G. W. Thorn and Dr. L. Wilkins for making these studies possible.

The level of aldosterone in blood of normal men is so low that very large blood samples are required (36). A considerable diurnal variation is seen (fig. 2) in the output of aldosterone in urine of normal men and women; and the timing of the cycle varies in different subjects, as Venning has also observed (37). The aldosterone content of 24-hour urine collections has been quite consistent in each individual despite variations in urine output, so long as the electrolyte intake and state of hydration are constant. Even in cases with marked, chronic impairment of renal function, the daily output of aldosterone is usually normal so long as the circulation and hydration are not disturbed.

Axelrad, Cates, Johnson, and Luetscher (38) found that acidification to pH 1.0 for 24 hours liberated sufficient aldosterone from urine of normal men to allow accurate determination. As the aldosterone content of urine increased during sodium deprivation or disease, considerable quantities of aldosterone were also found in extracts made from urine immediately after acidification to pH 1 and in urine incubated with β-glucuronidase. Venning, Genest, and their co-workers have made similar observations (39, 40).

Normal adults excrete 1 to 3 micrograms of aldosterone each day (23). Children show slightly smaller output. Aldosterone is not found in the urine of patients with Addison's disease, or after bilateral adrenalectomy, but most patients with hypopituitarism or hypophysectomy excrete normal amounts of aldosterone.

It is not possible at this time to make a final assessment of the effect of corticotropin (ACTH) on aldosterone output. Neither Venning (40) nor Axelrad (41) found significant changes after large doses of ACTH in normal men. Some patients under treatment with ACTH show increases of



18-ALDEHYDO-CORTICOSTERONE 11,18-HEMIACETAL

FIG. 1. Structure of aldosterone.

decreases in aldosterone excretion, which may be related to effects on their disease. For example, when a patient with nephrosis receives ACTH for 1–2 weeks, there is first an increase and later a decrease in aldosterone output, which can be correlated with changes in sodium balance (6).

In addition to possible effects on aldosterone output, ACTH may stimulate the secretion of unidentified material which increases diuresis and sodium excretion in the bioassay (42). Normal men excrete small amounts of material with similar activity (43, 44). The existence of an antagonist to desoxycorticosterone has been postulated to explain sodium loss during ACTH administration in children with adrenal virilism (45). The presence of an antagonist to aldosterone might explain our unexpected finding of normal levels of aldosterone in urine of some children with the sodium-losing syndrome associated with congenital adrenal hyperplasia.

There is general agreement on the high level of sodium-retaining activity in the urine of patients with the nephrotic syndrome, heart failure, hepatic cirrhosis, and toxemia of pregnancy (5–7, 14, 24, 38, 42, 46–54). In each of these diseases, the sodium-retaining factor has the typical chromatographic and pharmacologic properties of aldosterone (38). The quantity of aldosterone found in the urine is inversely correlated with sodium output (53, 54). In each disease, increased aldosterone output and accumulation of edema reflect exacerbation of the underlying disease, but the mechanism of increased secretion or decreased inactivation (55–57) remains uncertain.

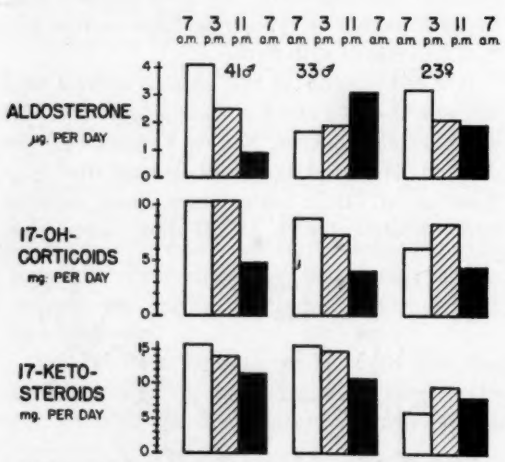


FIG. 2. Diurnal variation of aldosterone in urine of three normal subjects. A cycle differing from that of 17-hydroxy-corticoids (SILBER AND PORTER. *J. Biol. Chem.* 210: 923, 1954) and 17-ketosteroids is shown.

Restriction of sodium intake is followed by increased aldosterone output, which mirrors the fall in urine sodium (58). Sodium loading, resulting in output of sodium of over 500 milliequivalents per day, is followed by disappearance of measurable aldosterone from urine. Observations on five normal men and women, whose diets were held constant except for variations in sodium intake, are shown in figure 3. The inverse correlation between urine sodium and aldosterone is evident.

Large changes in potassium intake may also affect aldosterone output. Singer and Stack-Dunne (59) found that after potassium depletion, secretion of aldosterone by the rat adrenal was diminished. In patients, depletion sufficient to lower the serum potassium to 1.5–2.5 mEq/l. may be associated with marked reduction in aldosterone in urine. When depletion of potassium, sodium, and water occurs simultaneously, aldosterone in urine may be increased. In normal man, increased potassium intake (100 mEq KCl added to daily intake) is followed by diuresis, sodium loss, and a considerable rise in urine aldosterone (54).

These observations suggest that the effect of potassium on aldosterone is opposite to that of sodium, and that interaction may be important. Data from five normal men and women on intakes varying widely in sodium and potassium show that the quantity of aldosterone extracted from urine may be satisfactorily correlated with potassium to sodium ratio in that same collection of urine (fig. 4). Since both man and the rat respond to exogenous aldosterone with increased urine K/Na ratio (15, 22), the observed correlation between urine aldosterone and urine K/Na ratio adds further support to the hypothesis that the quantity of active hormone extracted from urine reflects the effective level of aldosterone in the body.

Recently, Conn, Mader, and Iseri (60, 61) have reported cases with adrenal tumor secreting large amounts of aldosterone. Similar findings have been attributed to 'potassium-losing nephritis' which may be associated with excessive production of aldosterone (62, 63). These patients show

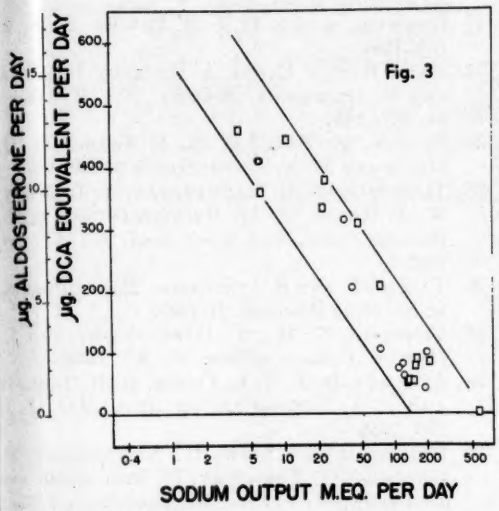


FIG. 3. Relationship between aldosterone and sodium in urine of normal subjects on different intakes of sodium.

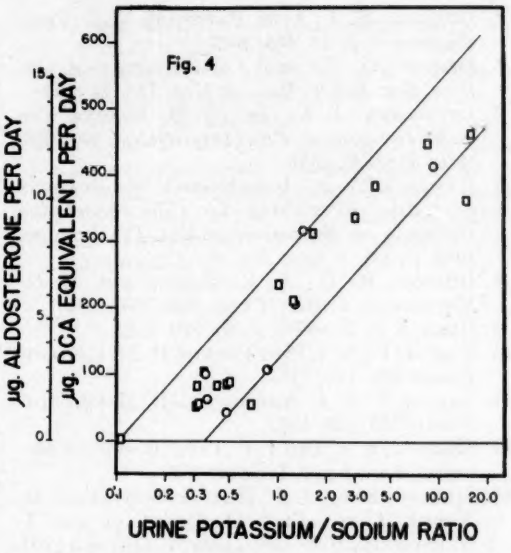


FIG. 4. Relationship between output of aldosterone and potassium to sodium ratio in urine of normal subjects on different intakes of sodium and potassium.

excessive potassium loss in urine, low serum potassium, alkalosis, and hypernatremia, which are reflected clinically in weakness, paralysis, or tetany of skeletal muscle, and abnormalities of cardiac and renal function related to potassium depletion (64–67). These cases represent a new class of adrenal cortical tumor, which differ from the adrenogenital syndrome and Cushing's syndrome in the chief hormone secreted, in metabolic pattern, and in clinical manifestations. Some overlap may occur between 'primary aldosteronism' and Cushing's syndrome, in which increased amounts of aldosterone sometimes occur. Furthermore, the possible role of corticosterone in 'primary aldosteronism' needs further investigation, since increased amounts of corticosterone were found in the tumor in Mader and Iseri's case.

SUMMARY

Aldosterone is the most potent sodium-retaining and potassium-eliminating hormone found in adrenal cortical extract, blood, and urine. Its secretion does not appear to be dependent upon pituitary corticotropin in the same degree as hydrocortisone and adrenal androgen.

Aldosterone output in urine is considered to reflect the circulating level of active hormone. Three general clinical patterns are associated with significant changes in aldosterone output: *1*) Alterations of dietary sodium and potassium in normal men are followed by changes in aldosterone output which suggest a homeostatic response. *2*) Exacerbation of heart failure, nephrotic syndrome, hepatic cirrhosis, or toxemia of pregnancy is associated with increased aldosterone output and retention of an excess of sodium and water by the kidneys. These phenomena are presumably secondary to the underlying disease, and the response to aldosterone must be conditioned by other physiological disturbances. *3*) In Addison's disease or after bilateral adrenalectomy, aldosterone is not found in urine. Adrenal cortical tumors which secrete large quantities of aldosterone may lead to severe potassium depletion and alkalosis, with resulting functional and anatomical changes in skeletal and cardiac muscle and in the kidneys.

REFERENCES

1. WINTERSTEINER, O. AND J. J. PFIFFNER. *J. Biol. Chem.* 116: 291, 1936.
2. KENDALL, E. C., H. L. MASON, W. J. HOELN AND B. F. MCKENZIE. *J. Biol. Chem.* 119: lvi, 1937.
3. KUIZENGA, M. H. AND G. F. CARTLAND. *Endocrinology* 24: 526, 1939.

4. Dorfman, R. I., A. M. Potts and M. L. Feil. *Endocrinology* 41: 464, 1947.
5. Deming, Q. B. and J. A. Luetscher, Jr. *Proc. Soc. Exper. Biol. & Med.* 73: 171, 1950.
6. Luetscher, J. A., Jr., Q. B. Deming and B. B. Johnson. *J. Clin. Investigation* 29: 1576, 1950; 30: 1530, 1951.
7. Hyman, E. S. Unpublished observations quoted by Luetscher. In: *Ciba Foundation Colloquia on Endocrinology* Vol. IV. London, 1952, p. 541.
8. Burton, R. B., A. Zaffaroni and E. H. Keutmann. *J. Biol. Chem.* 188: 763, 1951.
9. Bush, I. E. *Biochem. J.* 50: 370, 1952.
10. Tait, J. F., S. A. Simpson and H. M. Grundy. *Lancet* 262: 122, 1952.
11. Tait, J. F., S. A. Simpson and H. M. Grundy. *Nature* 169: 795, 1952.
12. Simpson, S. A. and J. F. Tait. *Memoirs Endocrinol. Soc.*, no. 2, London, 1953.
13. Simpson, S. A., J. F. Tait, A. Wettstein, R. Neher, J. von Euw, O. Schindler and T. Reichstein. *Helv. chim. acta* 37: 1163 and 1200, 1954.
14. Luetscher, J. A., Jr. and B. B. Johnson. *J. Clin. Investigation* 32: 585, 1953; 33: 276, 1954.
15. Luetscher, J. A., Jr. and B. B. Johnson. *Recent Progress in Hormone Research.* In press.
16. Luetscher, J. A., Jr., R. Neher and A. Wettstein. *Experientia* 10: 456, 1954.
17. Luetscher, J. A., Jr., R. Neher and A. Wettstein. *J. Biol. Chem.* In press.
18. Johnson, B. B. *Endocrinology* 54: 196, 1954.
19. Kagawa, C. M., E. G. Shipley and R. K. Meyer. *Proc. Soc. Exper. Biol. & Med.* 80: 281, 1952.
20. Marcus, F., L. P. Romanoff and G. Pincus. *Endocrinology* 50: 286, 1952.
21. Simpson, S. A. and J. F. Tait. *Endocrinology* 50: 150, 1952.
22. Singer, B. and E. H. Venning. *Endocrinology* 52: 623, 1953.
23. Luetscher, J. A., Jr. and B. J. Axelrad. *J. Clin. Endocrinol. & Metab.* 14: 1086, 1954.
24. Neher, R. and A. Wettstein. *Acta endocrinol.* In press.
25. Desaulles, P., J. Tripod and W. Schuler. *Schweiz med. Wchnschr.*, 83: 1088, 1953.
26. Sala, G. and J. A. Luetscher, Jr. *Endocrinology* 55: 516, 1954.
27. Renzi, A. A., M. Renzi, J. J. Chart and R. Gaunt. *J. Clin. Endocrinol. & Metab.* (Proc. Endocrine Society) 1955.
28. Gaunt, R., A. S. Gordon, A. A. Renzi, J. Padawer, G. J. Fruhman and M. Gilman. *Endocrinology* 55: 236, 1954.
29. Ward, L. E., H. F. Polley, C. H. Slocumb, P. S. Hench, H. L. Mason, V. R. Mattox and M. H. Power. *Proc. Staff Meet. Mayo Clin.* 15: 133, 1940.
30. Selye, H. and G. Heuser. *Fourth Annual Report on Stress.* Montreal: Acta, 1954, p. 72.
31. Desaulles, P., W. Schuler and R. Meier. *Experientia* 11: 68, 1955.
32. Kekwick, A. and G. L. S. Pawan. *Lancet* 2: 162, 1954.
33. Mach, R. S., J. Fabre, A. Duckert, R. Borth and P. Ducommun. *Schweiz. med. Wchnschr.* 84: 407, 1954.
34. Prunty, F. T. G., R. R. McSwiney, I. H. Mills and M. A. Smith. *Lancet* 2: 620, 1954.
35. Thorn, G. W., R. H. Sheppard, W. I. Morse, W. J. Reddy, P. M. Beigelman and A. E. Renold. *Ann. New York Acad. Sci.* 61: 609, 1955.
36. Tait, J. F. and S. A. Simpson. *Recent Progress in Hormone Research.* In press.
37. Venning, E. H., I. Dyrenfurth and C. Giroud. *Federation Proc.* 14: 297, 1955.
38. Axelrad, B. J., J. E. Cates, B. B. Johnson and J. A. Luetscher, Jr. *Brit. Med. J.* 1: 196, 1955.
39. Genest, J., E. Koiw, W. Nowacynski, M. Goldner, G. Tremblay, R. Robillard and L. Adamkiewicz. *Proc. Montreal Physiol. Soc.:* Symposium on Aldosterone, 1955.
40. Venning, E. H., I. Dyrenfurth, C. J. P. Giroud and J. C. Beck. *Proc. Montreal Physiol. Soc.:* Symposium on Aldosterone, Jan. 1955.
41. Axelrad, B. J., B. B. Johnson and J. A. Luetscher, Jr. *J. Clin. Endocrinol. & Metab.* 14: 783, 1954.
42. Luetscher, J. A., Jr. *Renal Function: Trans. 5th Conference.* New York: Macy, 1954, p. 22.
43. Luetscher, J. A., Jr. and B. B. Johnson. *J. Clin. Investigation* 33: 276, 1954.
44. Vanatta, J. C., W. W. Burr, Jr. and I. Danhof. *Arch. Biochem. Biophys.* 51: 309, 1954.
45. Jailer, J. W., J. Louchart and G. F. Cahill. *J. Clin. Investigation* 31: 880, 1952.
46. Chart, J. J. and E. G. Shipley. *J. Clin. Invest.* 32: 560, 1953.
47. Chart, J. J., E. G. Shipley and E. S. Gordon. *Proc. Soc. Exper. Biol. & Med.* 78: 244, 1951.
48. Gordon, E. S., J. J. Chart, D. Hagedorn and E. G. Shipley. *Obst. & Gynec. Surv.* 4: 39, 1954.
49. McCall, M. F. and B. Singer. *J. Clin. Endocrinol. & Metab.* 13: 1157, 1953.
50. Pechet, M. M., L. E. Duncan, Jr., G. W. Liddle and F. C. Bartter. *J. Clin. Investigation* 33: 957, 1954.
51. Singer, B. and J. Wener. *Am. Heart J.* 45: 795, 1953.
52. Venning, E. G., B. Singer and G. A. Simpson. *Am. J. Obst. & Gynec.* 67: 542, 1954.
53. Luetscher, J. A., Jr. and B. B. Johnson. *J. Clin. Investigation* 33: 1441, 1954.
54. Luetscher, J. A., Jr. and R. H. Curtis. *Ann. Int. Med.* In press.
55. Chart, J. J. Thesis. Univ. Wisconsin, 1954.
56. Gordon, E. S. *J. Lab. & Clin. Med.* 44: 80, 1954.

57. Reaven, G. M. *Endocrinology*. In press.
58. Luetscher, J. A., Jr. and B. J. Axelrad. *Proc. Soc. Exper. Biol. & Med.* 87: 650, 1954.
59. Singer, B. and M. P. Stack-Dunne. *J. Endocrinol.* 12: 130, 1955.
60. Conn, J. W. *J. Lab. & Clin. Med.* 45: 3, 1955.
61. Mader, I. J. and L. T. Iseri. *J. Lab. & Clin. Med.* 44: 895, 1954.
62. Evans, B. M. and M. D. Milne. *Brit. Med. J.* 2: 1067, 1954.
63. Cope, C. L. and J. Garcia-Llaurado. *Brit. Med. J.* 1: 1290, 1954.
64. Ferrebee, J. W., D. Parker, W. Carnes, M. Gerity, D. Atchley and R. Loeb. *Am. J. Physiol.* 135: 230, 1942.
65. Follis, R. H. *The Pathology of Nutritional Disease*. Springfield: Thomas, 1948, p. 32.
66. Bellet, S., W. A. Steiger, C. S. Nadler and P. C. Gazes. *Am. J. Med. Sci.* 219: 542, 1950.
67. Schwartz, W. B. and A. S. Relman. *J. Clin. Investigation* 32: 258, 1953.

AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS

Symposium on Cholesterol Metabolism[1]

Chairman: S. GURIN

BIOSYNTHESIS OF SUBSTANCES WHICH ACCOMPANY CHOLESTEROL[2]

ERWIN SCHWENK, GEORGE J. ALEXANDER, CHARLES A. FISH AND THOMAS H. STOUDT

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts and the Research Laboratories, Chemical Division, Merck & Company, Rahway, New Jersey

USING DEUTERIUM in experiments with rats, Waelsch, Sperry and Stoyanoff (1) observed that besides labeled cholesterol other substances occur in the non-saponifiable lipoids which have a higher isotope content than the cholesterol. They believed these substances to be alcohols and precursors of fatty acids. Schwenk and Werthessen (2) perfused acetate-C^{14} through pig livers and found similar results.

The substances accompanying cholesterol in such experiments may belong to the following groups: *1*) Precursors formed from acetate but not completely used up in the biosynthetic process, because of the steady-state nature of the biosynthesis of cholesterol in animals. They reveal their nature as precursors by being converted to cholesterol when they are isolated and fed to the animal and usually show a higher specific activity than the end product. *2*) Substances derived from cholesterol by enzymatic or chemical reactions, like oxidation with oxygen circulating in the blood. *3*) Substances which are endpoints of biosynthesis like cholesterol itself. The experiments show that all these are encountered.

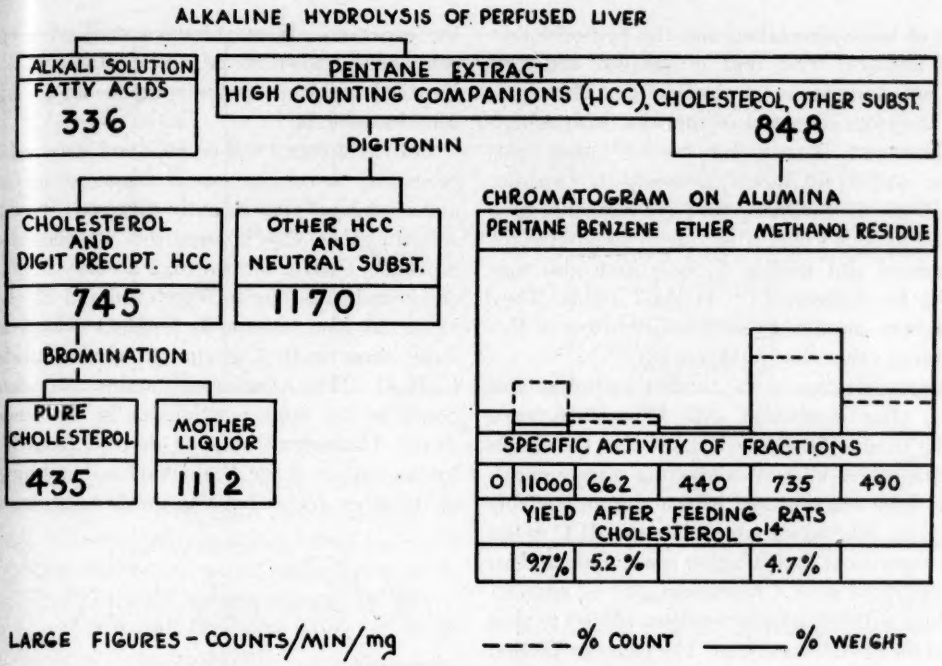
Figure 1 demonstrates the results of the perfusion of pig livers with acetate-C^{14} (3). After perfusion, liver was hydrolyzed with alkali and extracted with pentane, the fatty acids were then carefully eliminated and the residue of the pentane solution obtained in dry form. It consisted of the non-saponifiable lipoids, with a specific count of 848, which among other substances included the synthesized cholesterol. For the total of the radioactive substances which accompany cholesterol-C^{14} in these non-saponifiable lipoids the name 'higher counting companions' (HCC) was suggested. When this material was precipitated with digitonin, cholesterol-C^{14} of a specific count of 745 was obtained from the digitonide. The mother liquors from this precipitation still contained most of the HCC and a large part of the radioactivity. Recrystallization of the crude cholesterol obtained from the digitonide readily gave a substance of customary purity as shown by melting point, rotation, and infrared spectrum. Further recrystallizations, acetylation and chromatography did not change these properties, but the specific activity of this substance decreased with each purification step. This was caused by the presence of higher counting substances which were not eliminated by the normal purification procedures. Only dibromination and reconversion gave a cholesterol with a constant specific activity of 435, much lower than before, which did not change even when the bromination procedure was repeated.

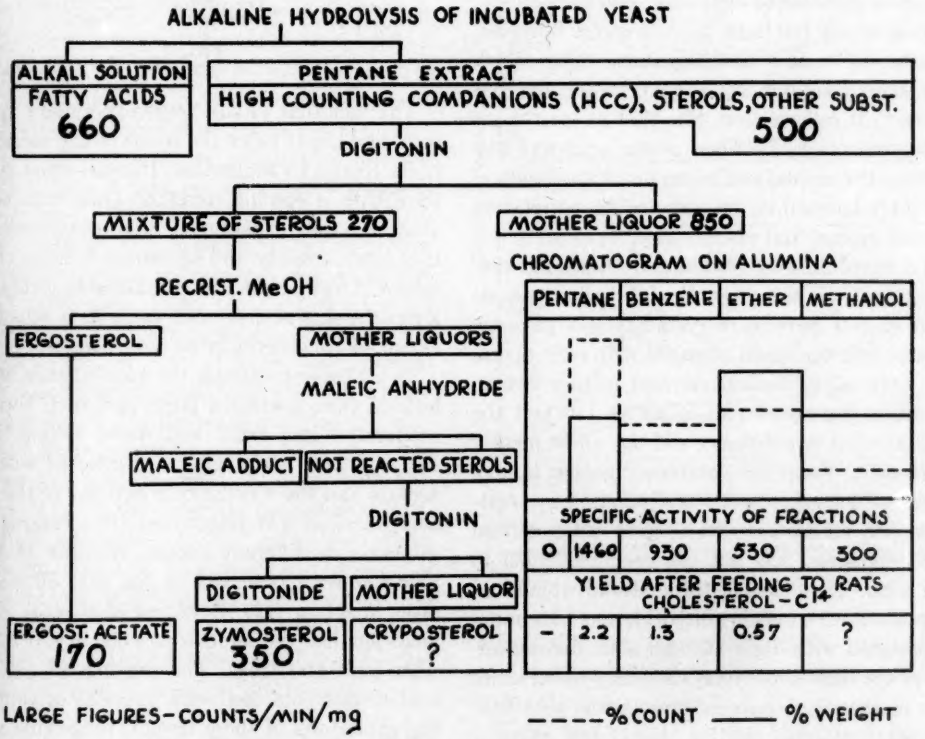
The digitonin precipitation revealed the presence in the HCC of a fraction (digitonin-precipitable HCC) which is not cholesterol but is precipitable with digitonin suggesting that this part of the HCC may be of sterol nature (2).

---

[1] San Francisco, California, April 14, 1955.

[2] This work has been supported by the Damon Runyon Fund, the Public Health Service (Grant No. C321), an institutional grant from the American Cancer Society, The Jane Coffin Childs Memorial Fund and the Schering Corporation, Bloomfield, N. J.





FIGS. 1 and 2

Chromatography on alumina of the non-saponifiable lipoids separated them into several fractions. Pentane first eluted partly crystallizing hydrocarbons of the C_{18} series with no radioactivity. The next eluates still with pentane, however, had a high specific count. After rechromatographing, the colorless oily hydrocarbon gave a solid hydrochloride. Analysis and infrared spec-

trum of the hydrocarbon and the hydrochloride were identical with that of purified standard squalene and its hexahydrochloride. This fraction therefore consisted of squalene with a high specific count. It gave cholesterol-C^{14} after feeding to rats (3) as shown previously by Langdon and Bloch (4). Benzene, ether and methanol gave other fractions which after careful elimination of cholesterol and feeding to rats, were also converted to cholesterol-C^{14} in good yields. They must have contained therefore precursors of this substance other than squalene (3).

An investigation of the alkaline hydrolysate of yeast, after incubation with C^{14}-acetate, gave similar results (5). Figure 2 shows that the sterols (in this case a mixture containing mainly ergosterol) were accompanied in the non-saponifiable lipoids by substances, which, like the HCC in the liver experiment, had a higher specific count than the ergosterol itself. Chromatography on alumina revealed a distribution of fractions similar to that found for the liver material. The pentane fraction was again squalene of high count and high purity. Feeding of all fractions to rats gave, however, cholesterol-C^{14} and not ergosterol into which these same fractions would have been converted by yeast. It follows from this that at least some of the precursors between acetic acid and the sterols in the animal and in the plant are identical and interchangeable; an interesting correlation between animal and plant.

The sterol mixture obtained by digitonin precipitation from the yeast lipoids had a specific count of 270. Several recrystallizations gave ergosterol, but the count changed with each operation. Only after acetylation and further recrystallization the count had fallen to 170 and the small amount of substance did not allow further purification. From the combined mother liquors the remaining ergosterol was eliminated by treatment with maleic anhydride, as an adduct from which ergosterol has not yet been reclaimed in pure form. That part of the material which had not reacted with maleic anhydride was once more precipitated with digitonin and after decomposition of the digitonide, recrystallized several times from methanol. A pure substance was obtained; infrared spectrum, melting point, and rotation proved it to be identical with an authentic sample of zymosterol. The count of this substance was 350, at least twice that of the ergosterol. The mother liquor of zymosterol gave material of questionable purity but still counting higher than the ergosterol. It may have contained cryptosterol also known as Δ^8,Δ^{24}-lanostadienol-3 (6) identical with one of the components of lanosterol in wool fat.

Both zymosterol and cryptosterol were isolated previously as companions of ergosterol in yeast, and Ruzicka (7) has recently suggested these two substances as direct precursors of cholesterol. Squalene, $C_{30}H_{50}$, formed from acetate, might be condensed according to Woodward and Bloch (8) to Δ^8,Δ^{24}-lanostadienol-3, $C_{30}H_{50}O$ which may loose three methyl groups to give zymosterol $C_{27}H_{44}O$. This substance contains two double bonds in the same positions as in the lanostadienol. Cholesterol may be derived from it by hydrogenation of the Δ^{24} double bond and moving of the other double bond to the Δ^5 position.[3]

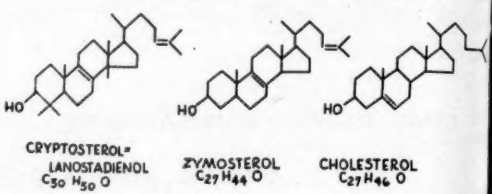


FIG. 3

The fact that in the yeast experiment zymosterol had about twice the count of ergosterol supports Ruzicka's suggestion. Indeed, when zymosterol with a specific count of 1400, obtained in another yeast experiment, was administered to rats intravenously and by stomach tube, cholesterol-C^{14} was isolated in considerable yields (11). Zymosterol, therefore, can serve as a direct precursor of cholesterol in its biosynthesis.

In all experiments on the biosynthesis of cholesterol from acetate a large part of C^{14} was incorporated into fatty and water soluble acids. For instance, when rats were injected with C^{14}-acetate and the specific radioactivity of the fatty acid fractions was determined after varying time intervals in different tissues, maxima of incorporation were observed in the first 30 minutes after injection (12). Beekman *et al.* (13) several years ago had the same experience in experiments with mice and frogs. The maxima of the fatty acid counts coincided with maxima of counts in the cholesterol of these tissues, suggesting a possible relationship of the two facts. When the methyl or ethyl esters of these acids were frac-

[3] That the Δ^8 double bond may be moved in the lanosterol series to the Δ^7 position was shown by Ruzicka *et al.* (9; cf. 10).

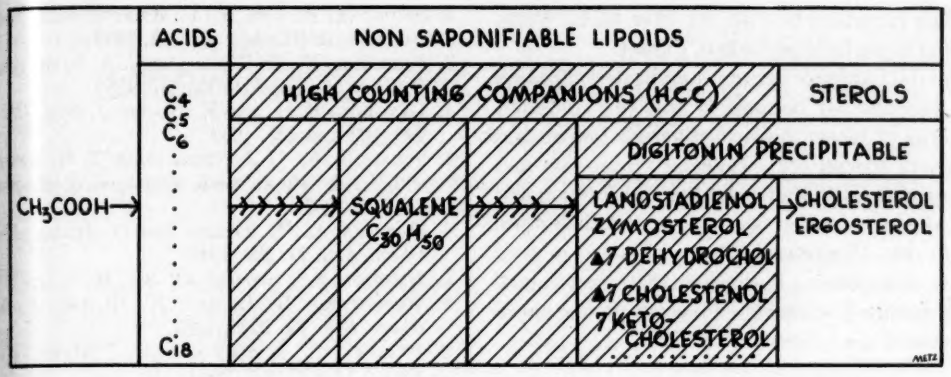


FIG. 4

tionated in vacuo the lower boiling fractions 180–200°/0.3 mm had about three times the count of the stearic acid which was isolated from the highest boiling fractions. Feeding experiments with or injection of the low boiling esters or the corresponding sodium salts however gave not more than traces of cholesterol-C^{14}.[4]

The facts known about the conversion of acetate into sterols may be presented as in figure 4. Acetate is first converted into acids with four to six carbon atoms as recently shown by several groups (compare other papers in this symposium). Squalene appears to be an identifiable intermediate, although it may not be a direct precursor of cholesterol (14). The fractions of the HCC which are eluted with benzene, ether and methanol from the alumina column have not yet yielded defined substances. From the digitonin precipitable HCC 3,5-dihydroxy-6-ketocholestane was previously obtained (15). It seems probable that it was derived from cholesterol after its biosynthesis because it had the same count as this substance. 7-Ketocholesterol and at least one other keto alcohol were recently obtained from this fraction (11). In the digitonin precipitable HCC one would also expect to find zymosterol which was shown above to be a possible precursor of cholesterol and Δ^8,Δ^{24}-lanostadienol-3 reported as precursor by Drs. Bloch and Clayton during this meeting. 7-Dehydrocholesterol and Δ^7-cholestenol may also be found in this fraction.

[4] Feeding or injecting the sodium salt or the ethyl ester of geranic acid $C_{10}H_{16}O_2$ labeled with C^{14} in the carboxyl group and prepared for us by Dr. M. Gut of this Institute gave no cholesterol-C^{14}. The water soluble acids in the experiments discussed, possibly containing acids with less than eight carbon atoms, have not yet been investigated.

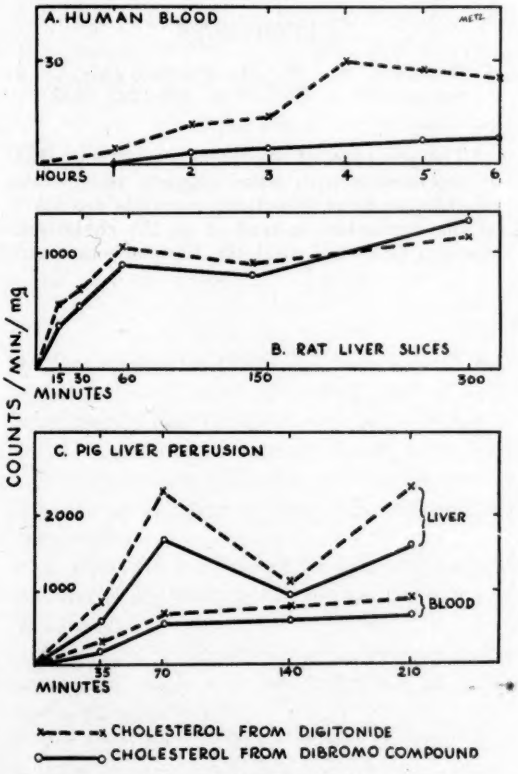


FIG. 5

The digitonin precipitable HCC are useful tools in the study of the biosynthesis of cholesterol, as shown in figure 5. (11)

When human, pig or rat blood is incubated with labeled acetate, cholesterol is synthesized in small amounts. In time experiments as presented in *Curve A* it can be seen that digitonin precipitable HCC are formed and that their specific counts decline with time while the specific counts of cholesterol increase. A similar picture is obtained

in time experiments with rat liver slices, *Curve B*,[5] and in pig liver perfusions, *Curve C*.

In intact animals a more complicated situation was encountered because of the very different patterns of biosynthesis in different tissues and in divers species (12). However, from rats and guinea pigs HCC were collected and fractionated and after elimination of squalene and cholesterol fed to rats. Cholesterol-C^{14} was obtained in good yields, thus proving that also in the intact animal as in perfused livers and in yeast precursors of cholesterol are present in the HCC.

Acknowledgment is made to Mr. D. Stevens for help with the biological experiments.

REFERENCES

1. Waelsch, H., W. M. Sperry and V. A. Stoyanoff, *J. Biol. Chem.* 135: 293, 1940.
2. Schwenk, E. and N. T. Werthessen, *Arch. Biochem. & Biophys.* 40: 334, 1952.
3. Schwenk, E., D. Todd and C. A. Fish. *Arch. Biochem. & Biophys.* 49: 187, 1954.
4. Langdon, R. G. and K. Bloch. *J. Biol. Chem.* 200: 129 and 135, 1952.
5. Schwenk, E., G. J. Alexander, T. H. Stoudt and C. A. Fish. *Arch. Biochem. & Biophys.* 55: 274, 1955.
6. Ruzicka, L., R. Denss and O. Jeger. *Helv. Chim. Acta* 29: 204, 1946.
7. Ruzicka, L. *Experientia* 9: 357, 1953.
8. Woodward, R. B. and K. Bloch. *J. Am. Chem. Soc.* 75: 2023, 1953.
9. Ruzicka, L., E. Rey and A. C. Muhr. *Helv. Chim. Acta* 27: 472, 1944.
10. Tschesche, R. and G. Snatzke. *Chem. Ber.* 88: 511, 1955.
11. Schwenk, E., G. T. Alexander, C. A. Fish and T. H. Stoudt. Unpublished observations.
12. Schwenk, E., G. J. Alexander and C. A. Fish. *Arch. Biochem. & Biophys.* In press.
13. Beekman, L. M. and G. de Eliott. *Nature* 167: 200, 1951.
14. Popjak, G. *Arch. Biochem. & Biophys.* 48: 102, 1954.
15. Schwenk, E., N. T. Werthessen and H. Rosenkrantz. *Arch. Biochem. & Biophys.* 37: 247, 1952.

[5] The presence of digitonin precipitable HCC in experiments with slices suggests that wrong results may be obtained where counts are taken on the digitonides instead of on the cholesterol after purification through the dibromo compound.

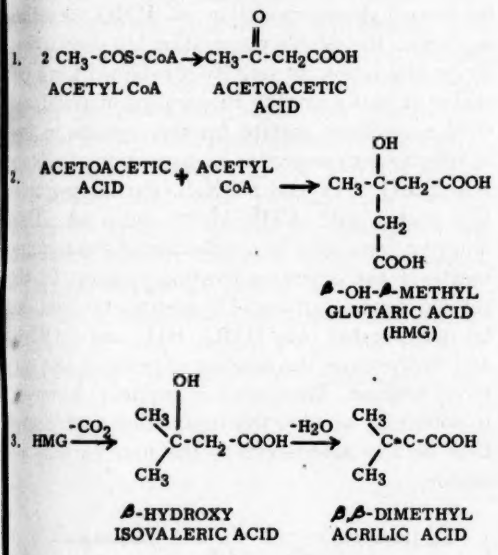
BIOSYNTHESIS OF BRANCHED CHAIN ACIDS[1]

HARRY RUDNEY AND TIBOR G. FARKAS

From the Department of Biochemistry, Western Reserve University School of Medicine, Cleveland, Ohio

RECENT work in our own, and the laboratories of the participants in this symposium (1–6) has served to outline the following sequence of reactions by which branched chain fatty acids such as β,β-dimethyl acrylic acid, β-hydroxy isovaleric and β-hydroxy-β-methyl glutaric acids are synthesized:

SCHEME 1


1. 2 CH3-COS-CoA → CH3-C(=O)-CH2COOH
ACETYL CoA
ACETOACETIC ACID
2. ACETOACETIC ACID + ACETYL CoA → CH3-C(OH)(CH2-COOH)-CH2-COOH
β-OH-β-METHYL GLUTARIC ACID (HMG)
3. HMG -CO2 → CH3-C(OH)(CH3)-CH2-COOH -H2O → CH3-C(CH3)=C-COOH
β-HYDROXY ISOVALERIC ACID
β,β-DIMETHYL ACRILIC ACID

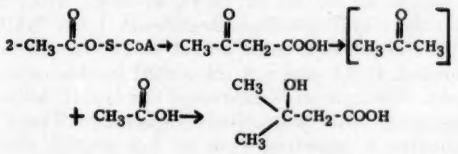
In this and succeeding schemes the acids are written in the free form, although it is realized that beyond acetyl CoA the acids are activated in the form of some acyl derivative such as an —SH group derivative. In our laboratory we have been interested in the various pathways by which β-hydroxy-β-methyl glutaric is synthesized and have attempted to assess their roles. There are several questions concerning this scheme about which there is no conclusive evidence, and upon which, some light may be thrown in this symposium.

1) Is the four carbon acceptor for acetyl CoA in *reaction 2* above free acetoacetic acid or acetoacetyl CoA?

2) What role does the CO_2 fixation reaction of Bachawat *et al.* (7) play in the synthesis of HMG[2]?

3) Has it been shown that the formation of a five carbon branched chain fatty acid according to Scheme 2 does not occur?

SCHEME 2



In this scheme the possibility is considered that some activated form of acetone derived from acetoacetate may condense with an acetyl derivative via the carbonyl group of acetone to form hydroxyisovaleric acid. This pathway represents a slight modification of that of Bonner and Arreguin (8).

4) What is the nature of the first condensation product of the five carbon unit on the pathway to squalene?

5) Finally, is CoA the cofactor involved in the subsequent steps after the activation of acetic and acetoacetic acid?

We have been able to obtain evidence concerning question 2 which shows that the synthesis of HMG is independent of CO_2 fixation and which indicates that the decarboxylation of HMG may be a different reaction from that involving the fixation of CO_2.

We have been studying a system which brings about the incorporation of labeled acetate into HMG, HIV, and β-OH-B. These compounds are

[1] These studies were aided by grants from the Life Insurance Medical Research Fund and the Elisabeth Severance Prentiss Fund of Western Reserve University.

[2] The following abbreviations are used: HMG = β-hydroxy-β-methyl glutaric acid; HIV = β-hydroxyisovaleric acid; β−OH−B = β-hydroxybutyric acid; CoA = coenzyme A; DMA = β,β-dimethyl acrylic acid.

TABLE 1

	Homogenate		Soluble Extract	
	1 $NaHC^{14}O_3$ + CH_3 COONa	2 $NaHCO_3$ $C^{14}H_3$ COONa	3 $NaHC^{14}O_3$ CH_3 COONa	4 $NaHCO_3$ $C^{14}H_3$ COONa
	cpm			
HMG	66	660	trace	1900
HIV	41	1,060	trace	9400
β-OH-B	3500	20,000	trace	2600

Each flask contained DPN 2.5 mg, ATP 2.5 mg, HMG 50 μM, HIV 50 μM, DMA 50 μM plus the following additions: *Flask 1*, 17.0 ml homogenate prep. + 20 μM $NaHC^{14}O_3$ + 10 μM CH_3COONa; *Flask 2*, 17.0 ml homogenate prep. + 20 μM $NaHCO_3$ + 10 μM $C^{14}H_3COONa$; *Flask 3*, 14.0 ml soluble prep. + 20 μM $NaHC^{14}O_3$ + 10 μM CH_3COONa; *Flask 4*, 14.0 ml soluble prep. + 20 μM $NaHCO_3$ + 10 μM $C^{14}H_3COONa$.

Specific activity of $NaHC^{14}O_3$, 10×10^6 cpm/μM; Specific activity of $C^{14}H_3COONa$, 5.5×10^4 cpm/μM.

Incubated for 2.5 hr. in O_2 at 38°C. After incubation, and alkaline treatment (1, 2) HMG, HIV and β-OH-B were added as carrier and reisolated. DMA was not reisolated in this experiment. Counts are expressed as total counts present in the respective compounds. 'Trace'—indicates a counting rate of 1–2 counts above background and hence not considered to be significant.

readily isolated for analysis by a sequence of ion exchange and partition chromatographic procedures. The following experiment was performed. Labeled acetate and unlabeled bicarbonate, and labeled bicarbonate and unlabeled acetate, were incubated with the required cofactors with two preparations capable of forming branched chain acids, one being a homogenate system (9) and the other a water-soluble preparation (9). After incubation the total counts incorporated into each acid were determined. It is assumed here that β-OH-B reflects the pattern of labeling in acetoacetic acid. Table 1 shows the results obtained in a representative experiment. These results show that in the homogenate preparation CO_2 fixation occurs mostly into β-OH-B and to a small but significant extent into HMG and HIV. Degradation of the β-OH-B revealed the isotope to be located solely in the carboxyl group. In the water-soluble preparation the CO_2 fixation reaction is essentially abolished since only a trace of $C^{14}O_2$ appears in the HMG, HIV, and β-OH-B. These results offer support for the postulate of Bachawat *et al.* (7) that CO_2 fixation into acetoacetic acid is dependent upon fixation into HMG, since when it is abolished in HMG it is also abolished in β-OH-B. It will be noted however, that although CO_2 fixation ceases in the water-soluble preparation, the formation of HIV and HMG from acetate is unimpaired. This indicates that the decarboxylation of HMG to HIV is a different reaction from the CO_2 incorporation into HMG, or that the position of equilibrium under the conditions used in the soluble enzyme experiments is far toward de-carboxylation of HMG. Further work with the soluble preparation has shown that by lyophilization its activity is retained for a period of at least 6 months. Preparation of a dialyzed 60% ammonium sulfate fraction results in loss of activity with respect to incorporation of acetate into HMG, HIV and β-OH-B. Partial reactivation occurs with ATP, Mg^{++} and CoA. This, however, may only be a reflection of the requirements of the acetate-activating system. In the soluble system carbonyl-C^{14} acetoacetic acid can be incorporated into HMG, HIV and β-OH-B and furthermore the labeling appears in the carboxyl position. More work is required, however, to determine whether this means that acetoacetyl CoA or free acetoacetic is the four carbon acceptor.

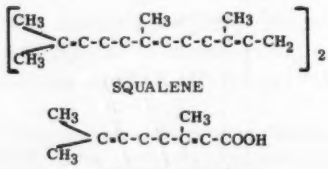


FIG. 1

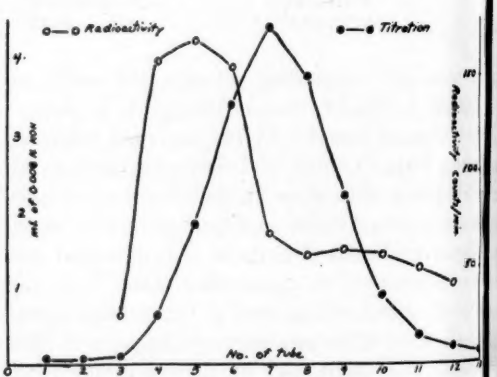


FIG. 2. Noncoincidence of radioactivity and titration value for geranic acid isolated on a Howard and Martin column, stationary phase paraffin, moving phase 55% w/v aqueous acetone. Volume of each tube was 3.0 ml.

Finally, we have attempted to determine the nature of the ten carbon unit which might result from the condensation of two molecules of activated DMA. As Dr. Bloch has shown, DMA is incorporated into cholesterol without apparent prior breakdown into two carbon units (5). As a starting point we have used the compound geranic acid derived from the oxidation of citral. Figure 1 shows how its carbon skeleton is related to squalene.

Geranic acid and methyl labeled acetic acid were incubated in a homogenate system which synthesized cholesterol from acetate (9). Acyl SH derivatives were hydrolyzed with alkali. Then more geranic acid was added as carrier and reisolated by the silica gel column technique of Vandenheuvel and Hayes (10) for the separation of long chain fatty acids. On this column considerable activity appeared in the geranic acid, roughly corresponding to the titration values. However, when the reversed phase chromatographic procedure of Howard and Martin (11) was used it was found that radioactivity and titration values did not coincide (fig. 2). Oxidation of the geranic acid with permanganate, which cleaves the terminal part of the branched chain to acetone, showed that no labeling was present in this portion of the geranic acid. Thus it appears that a compound, not geranic acid but resembling it with respect to certain physical characteristics, is formed from acetic acid. Essentially similar results were obtained in an experiment where geranic acid was not added during the incubation with acetate, but only at the end as carrier. It is obvious that an immense number of derivatives of geranic acid are possible and could account for the radioactive peak, and it is hoped that further work may indicate their nature. It also stresses the already obvious fact that rigid purification and identification procedures will be necessary to characterize these intermediates accurately since these compounds isomerize readily.

REFERENCES

1. Rudney, H. *J. Am. Chem. Soc.* 76: 2595, 1954.
2. Rudney, H. *J. Am. Chem. Soc.* 77: 1698, 1955.
3. Rabinowitz, J. L. *J. Am. Chem. Soc.* 76: 3037, 1954.
4. Rabinowitz, J. L. and S. Gurin. *J. Am. Chem. Soc.* 76: 5168, 1954.
5. Bloch, K., L. C. Clark and I. Harary. *J. Biol. Chem.* 211: 687, 1954.
6. Bonner, J., M. W. Parker and J. C. Montermoso. *Science* 120: 549, 1954.
7. Bachawat, B. K., W. G. Robinson and M. J. Coon. *J. Am. Chem. Soc.* 76: 3098, 1954.
8. Bonner, J. and B. Arreguin. *Arch. Biochem.* 21: 104, 1949.
9. Rabinowitz, J. L. and S. Gurin. *J. Biol. Chem.* 208: 307, 1954.
10. Vandenheuvel, F. A. and E. R. Hayes. *Anal. Chem.* 24: 960, 1952.
11. Howard, G. A. and A. J. P. Martin. *Biochem. J.* 46: 532, 1950.

BRANCHED CHAIN ACIDS IN THE BIOSYNTHESIS OF SQUALENE AND CHOLESTEROL[1]

JOSEPH L. RABINOWITZ, F. DITURI, F. COBEY AND S. GURIN

From the Department of Biochemistry, University of Pennsylvania, and Radioisotope Unit, Veterans Administration Hospital, Philadelphia, Pennsylvania

IT HAS BEEN SHOWN in several laboratories that isotopically labeled acetate can be converted by mammalian and plant tissues into five and six carbon branched chain acids such as β-hydroxy-β-methyl glutarate, (1, 2) β-methylcrotonate, (3–5) β-hydroxy isovalerate, (6) and trans-β-methyl glutaconic acid (3). There is also evidence that some or all of these may be involved as precursors of cholesterol (1, 2, 6, 7), squalene (7, 8) and rubber (5).

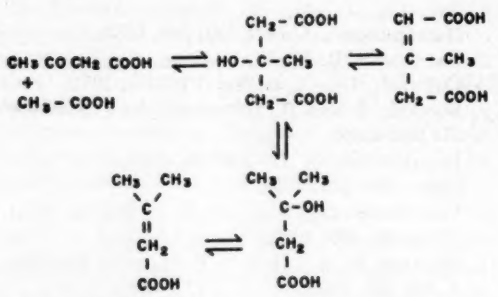


FIG. 1. Biosynthesis and interconversions of branched chain acids.

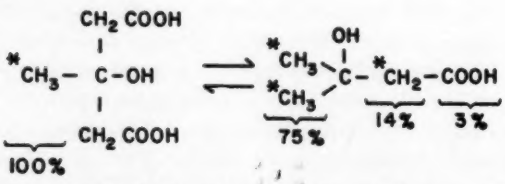


FIG. 2. Distribution of radioactivity in β-hydroxy isovalerate following incubation with labeled β-hydroxy-β-methyl glutarate.

Studies in this laboratory on the biosynthesis of squalene and cholesterol have been carried out with a particle free extract of rat liver. By combining an aqueous extract of mitochondria with supernatant fluid, a system is obtained which can incorporate labeled acetate or β-hydroxy-β-methyl glutarate into squalene (8) and cholesterol (1). While the supernatant fluid alone cannot incorporate acetate into squalene or cholesterol, it effectively converts labeled β-hydroxy-β-methyl glutarate into squalene (8).

Using labeled acetate as a substrate with a system containing mitochondrial extract and supernatant fluid, the addition of the above mentioned unlabeled branched chain acids resulted in a reduction in the incorporation of acetate into cholesterol. After incubation, the added acids were recovered and found to be radioactive. When labeled β-hydroxy β-methyl glutarate was used as a substrate, radioactivity could be found in β-methyl crotonate, β-hydroxy isovalerate, and trans-β-methyl glutaconic acid (1, 3, 4). Figure 1 shows some possible interconversions between

TABLE 1. INCORPORATION OF LABELED SUBSTRATES INTO SQUALENE BY SUPERNATANT ENZYMES

SUBSTRATE	CONCENTRATION	μM C^{14} INCORPORATED INTO SQUALENE
HMG*	30 μM	0.021
SA*	100 μM	0.007
HIV*	40 μM	TRACE

acetate, acetoacetate and these branched chain acids.

After incubation of mitochondrial extract and supernatant fluid with methyl labeled β-hydroxy-β-methyl glutaric acid and carrier β-hydroxy isovaleric acid, the hydroxy isovaleric acid was isolated and degraded (6). Figure 2 shows that the recovered acid was multiply labeled. This would imply that β-hydroxy-β-methyl glutaric acid is in part broken down to acetoacetate or active acetoacetate with a resulting redistribution of the isotope in acetoacetate and probably in the β-hydroxy β-methyl glutarate.

With supernatant enzymes alone, labeled β-hydroxy-β-methyl glutarate is incorporated readily into squalene, while β-methyl crotonate is incorporated less readily and β-hydroxy isovalerate only in trace quantities (table 1). No require

[1] Supported by grants of the Atomic Energy Commission and the National Heart Institute of the National Institutes of Health.

TABLE 2. INCORPORATION OF C^{14}-SQUALENE INTO CHOLESTEROL BY PARTICLE-FREE EXTRACTS

Exp.	Squalene $-C^{14}$	μM Squalene incorporated into cholesterol
No. 1	3500 cpm/μM	0.034
No. 2	860 cpm/μM	0.037

ment for coenzyme A has been shown in this system, nor has it been possible to demonstrate the incorporation of the CoA derivatives of the above named acids into squalene. It is probable that some form of activation is required, since β-methyl crotonic acid is utilized while β-hydroxy isovalerate is not.

It has been shown by Langdon and Bloch (9) that mammalian liver slices incorporate labeled acetate into squalene, and that the resulting squalene can be incorporated into cholesterol. We have prepared biosynthetic squalene of high specific activity by incubation of labeled acetate with mitochondrial enzymes supplemented with supernatant fluid. The resulting radioactive squalene was incubated with the combined mitochondrial extract-supernatant fluid system and labeled cholesterol was recovered (table 2).

Preliminary studies indicate that squalene may be synthesized biologically, as it is chemically, by the condensation of two farnesyl radicals (8). During the incubation of supernatant fluid with labeled β-hydroxy-β-methyl glutarate, the addition of carrier farnesol or farnesenic acid markedly reduces the incorporation of substrate into squalene. Upon recovery after incubation, the farnesol contained no radioactivity while the farnesenic acid was significantly labeled. Further studies are in progress.

REFERENCES

1. RABINOWITZ, J. L. AND S. GURIN. *J. Biol. Chem.* 208: 307, 1954.
2. RUDNEY, H. *J. Am. Chem. Soc.* 76: 2595, 1954.
3. RABINOWITZ, J. L. AND S. GURIN. *J. Am. Chem. Soc.* 76: 5168, 1954.
4. RABINOWITZ, J. L. *J. Am. Chem. Soc.* 76: 3037, 1954.
5. BONNER, J. AND B. ARREGUIN. *Arch. Biochem.* 21: 109, 1949.
6. RABINOWITZ, J. L. *J. Am. Chem. Soc.* 77: 1295, 1955.
7. BLOCH, J., L. C. CLARK AND I. HARARY. *J. Biol. Chem.* 211: 687, 1954.
8. DITURI, F., F. COBEY, J. V. B. WARMS AND S. GURIN. *Federation Proc.* 14: 203, 1955.
9. LANGDON, R. G. AND K. BLOCH. *J. Biol. Chem.* 200: 135, 1953.

ENZYMATIC SYNTHESIS OF BRANCHED CHAIN ACIDS FROM AMINO ACIDS[1]

M. J. Coon[2]

From the Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Since other reports at this symposium have emphasized the role of a branched chain monomer in the biosynthesis of complex organic molecules, it would seem particularly important to consider here the biological origin of simple branched chain structures. The investigations to be described, carried out in collaboration with Drs. B. K. Bachhawat, W. G. Robinson, and F. P. Kupiecki and Mr. R. Nagle, have established the synthesis of a variety of branched chain intermediates from amino acids in soluble enzyme preparations from animal tissues.

(*1*) α-Methylacetoacetate + succinyl CoA ⇌ α-methylacetoacetyl CoA + succinate

(*2*) α-Methylacetoacetyl CoA + CoA ⇌ acetyl CoA + propionyl CoA

Fig. 1. Enzymatic steps in isoleucine degradation.

As described earlier, compounds having the isoprene carbon skeleton are derived from the catabolism of isoleucine (1–3). α-Methylbutyryl CoA[3] is dehydrogenated in heart or liver extracts to furnish tiglyl CoA, which undergoes hydration, catalyzed by crotonase, and then oxidation in the presence of DPN to yield α-methylacetoacetyl CoA. It has recently been found that this β-keto thiol ester can also be formed by transfer of CoA to α-methylacetoacetate (*Reaction 1*, fig. 1), a process similar to the succinate-acetoacetyl CoA transfer known to occur in heart preparations (4, 5), and that acetyl CoA and propionyl CoA are subsequently liberated by a cleavage step (*Reaction 2*). Since the condensation of acetyl CoA units to furnish acetoacetyl CoA is known to occur readily, there is no reason to believe that the condensation of acetyl CoA and propionyl CoA to produce α-methylacetoacetyl CoA (by reversal of *Reaction 2*) may not also occur in animal tissues. Such a mechanism would provide a simple means of obtaining branched chain compounds from substrates readily available from carbohydrate and lipid catabolism.

Recently we have extended our work on branched chain thiol esters to compounds derived from valine, and have obtained evidence for the scheme in figure 2, which is an extension of those proposed by Atchley (6) and more recently by Kinnory, Takeda, and Greenberg (7). This scheme, based on enzymatic studies, is in accord with isotopic data obtained by other investigators (7–10). Isobutyryl CoA is dehydrogenated by enzyme fractions of heart and liver, as would be predicted by analogy to the corresponding dehydrogenation of the CoA thiol esters of straight chain fatty acids (11, 12). The resulting methacrylyl CoA is hydrated non-enzymatically to furnish β-hydroxyisobutyryl CoA, but the latter compound remains unchanged in our enzyme preparations unless the CoA is removed, in which case the free β-hydroxyisobutyrate is converted to methylmalonaldehydic acid by a specific DPN-dependent dehydrogenase. Preliminary data indicate that methylmalonyl CoA may be formed by oxidation of the aldehyde acid. The condensation of carbon dioxide with propionyl CoA to furnish methylmalonate, described at these meetings by Flavin (13), may represent the reversal of the final step in valine degradation. In the presence of glutamate, methylmalonaldehydic acid undergoes amination to furnish β-aminoisobutyrate, which is also known to arise from thymine (14).

Earlier isotopic studies on the degradation of

[1] Supported by grants from the National Science Foundation and the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

[2] Present address: Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor.

[3] The following abbreviations are used: coenzyme A, CoA; thiol ester of coenzyme A, acyl CoA; β-hydroxy-β-methylglutaric acid, HMG; β-hydroxy isovaleric acid, HIV; diphosphopyridine nucleotide, DPN; adenosine triphosphate, ATP; inosine triphosphate, ITP; tris (hydroxymethyl) aminomethane, Tris.

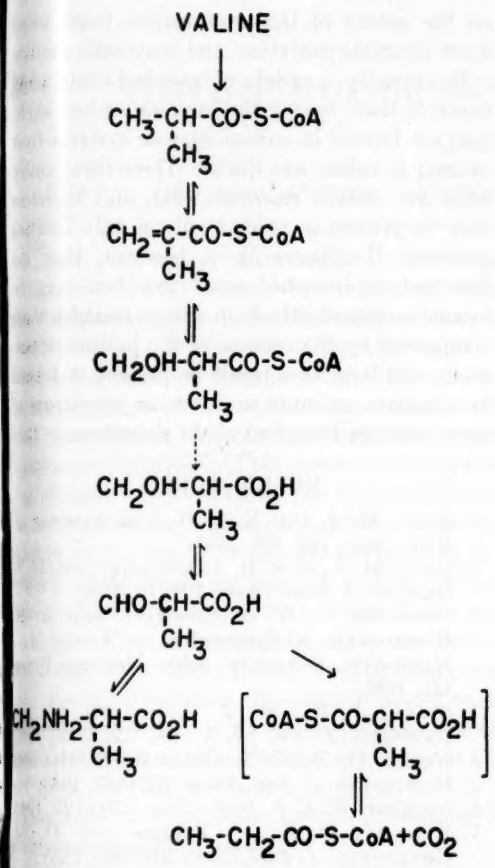


FIG. 2. Proposed scheme of valine and isobutyrate metabolism.

sovaleric acid, an intermediate in leucine metabolism, established two reactions unique in fatty acid metabolism—carbon dioxide fixation into the carboxyl position of acetoacetate, and cleavage of the branched carbon chain without prior formation of a β-keto acid (15–19). All attempts to obtain evidence for a hypothetical 3-carbon intermediate derived from the isopropyl group of isovalerate were unsuccessful, and more recent studies have led to the demonstration of the reaction sequence shown in figure 3 (3, 20, 21).

Isovaleric acid itself is inert, but in the form of its CoA thiol ester it is dehydrogenated to form β-methylcrotonyl CoA, and this compound is in turn hydrated by crotonase to furnish β-hydroxyisovaleryl CoA (HIV CoA). Although one would preduct that the methyl groups of the unsaturated compound would be much more reactive than those of the hydroxy compound, the carboxylase nevertheless chooses to react carbon dioxide with a methyl group of HIV CoA to

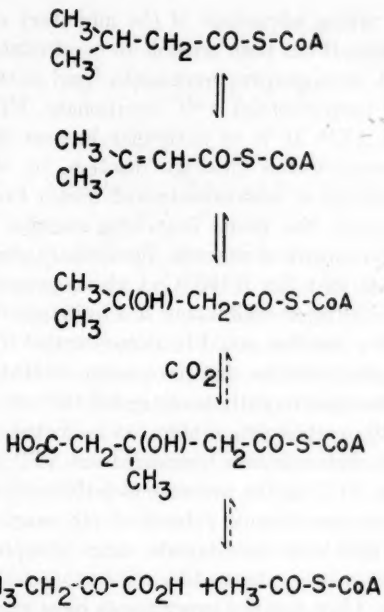


FIG. 3. Enzymatic steps in the conversion of isovaleryl CoA to acetoacetate.

TABLE 1. ENZYMATIC LIBERATION OF PYROPHOSPHATE FROM ATP IN THE PRESENCE OF BICARBONATE

Exper.	Substrates Added	Pyrophosphate Formed, μM
1	$KHCO_3$ + ATP	0.91
	$KHCO_3$ + ATP + HIV CoA	1.40
2	$KHCO_3$ + ATP + HIV CoA	0.53
	$KHCO_3$ + ITP + HIV CoA	0.02

The test system contained 13 μM of glutathione, 200 μM of potassium fluoride, 50 μM of magnesium chloride, dialyzed heart extract (8.2 mg of protein), and, where indicated, 500 μM of potassium bicarbonate, 10 μM of ATP, and 2 μM of HIV CoA; final volume, 3.0 ml; incubation time, 30 min. at 38°. Pyrophosphate was estimated colorimetrically (23), and the values reported are corrected for small amounts of pyrophosphate formed in control experiments in which 500 μM of Tris, pH 8.1, were substituted for bicarbonate.

produce the 6-carbon intermediate, β-hydroxy-β-methylglutaryl CoA (HMG CoA), which is rapidly cleaved between the α and β carbons to furnish equimolar amounts of acetoacetate and acetyl CoA. The cleavage enzyme, as reported at these meetings (22), requires the presence of magnesium or manganese ions and a thiol compound such as glutathione or cysteine, but is strongly inhibited by thiomalate or by malate.

By taking advantage of the inhibitory effect of malate, it has been possible to accumulate HMG CoA in an enzyme preparation freed of crotonase and supplemented with bicarbonate, HIV CoA, and ATP. It is of particular interest that the reversal of this cleavage reaction, by the condensation of acetoacetate and acetyl CoA, may represent the initial branching reaction leading to precursors of steroids. Preliminary studies indicate that the HMG CoA cleavage reaction is not readily reversible, but it is quite possible that such a reaction could be demonstrated if it were coupled with the decarboxylation of HMG CoA.

We have recently investigated the role of ATP in the carboxylation step. As indicated in table 1, pyrophosphate is liberated from ATP (but not from ITP) in the presence of a dialyzed alcohol-potassium chloride extract of pig heart supplemented with bicarbonate, and pyrophosphate liberation is enhanced by the further addition of HIV CoA. Control experiments have established that in the absence of bicarbonate, HIV CoA does not stimulate pyrophosphate release from ATP. Furthermore, in the absence of enzyme, no pyrophosphate is released from ATP by the addition of bicarbonate or HIV CoA alone or in combination.

(3) CO_2 + ATP $\rightleftharpoons$ Activated CO_2 + pyrophosphate

(4) Activated CO_2 + HIV CoA $\rightleftharpoons$ HMG CoA

FIG. 4. Proposed steps in carbon dioxide activation and fixation.

These results have led us to propose that the carboxylation reaction may involve the two distinct enzymatic steps shown in figure 4. *Reaction 3* represents the activation of carbon dioxide with the accompanying release of pyrophosphate. *Reaction 4* represents the condensation of HIV CoA with activated carbon dioxide to furnish HMG CoA, thereby pulling *Reaction 3* to the right and increasing pyrophosphate release. Preliminary experiments indicate that the proposed intermediate, although highly unstable, can be recovered in small amounts by chromatography at 4° in 50% ethanol on Whatman no. 3 paper. Adenosine phosphoryl carbonate is suggested as a possible structure for the intermediate, for such a compound would be expected to have a 'high energy' bond linking phosphate and carbonate and be capable of reacting with the relatively inert methyl groups of HIV CoA. A final decision on the nature of the intermediate must await more complete analytical and enzymatic studies.

In summary, a variety of branched chain acids, many of them having the isoprene carbon skeleton, are formed in animal enzyme systems from isoleucine, valine, and leucine. These three amino acids are dietary essentials (24), and therefore may be present in animal cells in only limiting amounts. It appears likely, however, that the intermediate branched acids described may be formed independently from simple straight chain compounds by the reversal of the pathways outlined, and may as a result be present in tissues in adequate amounts to serve as precursors of more complex branched chain structures.

REFERENCES

1. COON, M. J. AND N. S. B. ABRAHAMSEN. *J. Biol. Chem.* 195: 805, 1952.
2. COON, M. J., N. S. B. ABRAHAMSEN AND G. S. GREENE. *J. Biol. Chem.* 199: 75, 1952.
3. COON, M. J., W. G. ROBINSON AND B. K. BACHHAWAT. *A Symposium on Amino Acid Metabolism.* Baltimore: Johns Hopkins Press, 431, 1955.
4. STERN, J. R., M. J. COON AND A. DEL CAMPILLO. *J. Am. Chem. Soc.* 75: 1517, 1953.
5. GREEN, D. E., D. S. GOLDMAN, S. MII AND H. BEINERT. *J. Biol. Chem.* 202: 137, 1953.
6. ATCHLEY, W. A. *J. Biol. Chem.* 176: 123, 1948.
7. KINNORY, D. S., Y. TAKEDA AND D. M GREENBERG. *J. Biol. Chem.* 212: 385, 1955.
8. ROSE, W. C. Personal communication.
9. GRAY, I., P. ADAMS AND H. HAUPTMANN *Experientia* 6: 430, 1950.
10. FONES, W. S., T. P. WAALKES AND J. WHITE *Arch. Biochem. and Biophys.* 32: 89, 1951.
11. SEUBERT, W. AND F. LYNEN. *J. Am. Chem. Soc.* 75: 2787, 1953.
12. GREEN, D. E., S. MII, H. R. MAHLER AND R. M. BOCK. *J. Biol. Chem.* 206: 1, 1954.
13. FLAVIN, M. *Federation Proc.* 14: 211, 1955.
14. FINK, K. AND C. MCGAUGHEY. *Federation Proc* 13: 207, 1954.
15. BLOCH, K. *J. Biol. Chem.* 155: 255, 1944.
16. COON, M. J. AND S. GURIN. *J. Biol. Chem* 180: 1159, 1949.
17. ZABIN, I. AND K. BLOCH. *J. Biol. Chem.* 185 117, 1950.
18. COON, M. J. *J. Biol. Chem.* 187: 71, 1950.
19. PLAUT, G. W. E. AND H. A. LARDY. *J. Biol Chem.* 192: 435, 1951.
20. BACHHAWAT, B. K., W. G. ROBINSON AND M J. COON. *J. Am. Chem. Soc.* 76: 3098, 1954.
21. BACHHAWAT, B. K., W. G. ROBINSON AN M. J. COON. *J. Biol. Chem.* In press.
22. ROBINSON, W. G., B. K. BACHHAWAT AN M. J. COON. *Federation Proc.* 14: 270, 1955.
23. FLYNN, R. M., M. E. JONES AND F. LIPMAN *J. Biol. Chem.* 211: 791, 1954.
24. ROSE, W. C. *Physiol. Rev.* 18: 109, 1938.

SYNTHESIS OF BRANCHED CHAIN COMPOUNDS[1]

JAMES BONNER

From the Division of Biology, California Institute of Technology, Pasadena, California

SOME years ago we undertook to find out something about the pathway of formation of rubber by rubber-bearing plants. Rubber is an isoprenoid made up of repeating isoprene units as are the lower terpenes, sesquiterpenes, diterpenes and tetraterpenes. It differs from these lower members of the series in being a high molecular weight polymer, containing of the order of a hundred to a thousand isoprene units per linear chain. Our study of rubber formation has been made possible by the fact that we have available to us a plant Parthenium, the guayule, a native of Mexico, which synthesizes rubber abundantly and rapidly. This rubber is contained in the bark of the stems and the roots. Rubber formation occurs in the guayule when the plant is grown under conditions favorable for photosynthesis but unfavorable for increase in size of the plant (1). Under such circumstances, a major portion of the daily photosynthetic increment is stored as the rubber hydrocarbon.

The formation of rubber by the bark of seedling guayule plants may be increased by supplying them with a suitable substrate. Thus, it has been shown that net rubber formation is increased when seedling guayule plants are supplied with acetate (2). Furthermore, if the acetate supplied is labeled with C^{14} in both carbon atoms, the rubber synthesized by the plant possesses a specific activity equal to that of the acetate given (3, 4). It would appear, therefore, that every carbon atom in the rubber molecule can be channeled through and derived from acetate. A great many other compounds do not support the formation of rubber by guayule plants. One compound which possesses the five carbon branched chain was, however, found some years ago to be able to replace acetate in the function of supporting net rubber formation by guayule. This compound is the five carbon branched chain acid β-methylcrotonate (2). β-methylcrotonate not only supports the net formation of rubber in the intact guayule plant, but is some 5–10 times more effective in this function than is acetate itself. When β-methylcrotonate labeled with C^{14} is supplied to the plant, the rubber produced is found to be labeled. β-methylcrotonate may be derived from acetate in the plant, since β-methylcrotonate isolated from plants which have been supplied with labeled acetate is itself labeled (4).

In the study of the path of isoprenoid synthesis by the guayule we have two problems. The first is the path of carbon atoms from acetate to the isoprenoid structure which forms the monomer of the long chain molecule. This monomer may be β-methylcrotonate or some closely related derivative of it. A second problem is that of how the monomer units, β-methylcrotonyl units for example, are polymerized to form the long linear rubber molecule.

The facts available to us at the present time suggest that the path of acetate to β-methylcrotonate in the plant (5) follow the general outline shown in figure 1. In this pathway acetate is first converted to acetyl-CoA by the acetate-activating enzyme. Acetyl-CoA is then joined with itself to form the four carbon compound acetoacetyl-CoA which is then hydrolyzed to acetoacetate. This compound, acetoacetate, is next condensed with still another acetyl-CoA molecule to form a six carbon compound, β-hydroxy-β-methylglutaryl-CoA (BOG). The BOG CoA is then decarboxylated and dehydrated to form β-methylcrotonyl-CoA.

The key intermediate in this series of reactions, β-hydroxy-β-methylglutarate (BOG) was first suspected as an intermediate when it was tentatively identified as a product of the metabolism of C^{14}-labeled acetate by acetone powders of leaves (6). Since this observation in 1953, it has been shown by several groups that BOG is in fact a naturally occurring plant product (7, 8). The sequence of *reactions 1* to *4* of figure 1 can be carried out with an enzyme system from flax seedlings, a plant which accumulates BOG in its seeds. The enzymatic system consists of lysed

[1] Report of work supported in part by the Chemicals and Plastics Branch, Headquarters Research and Development Command, Office of the Quartermaster General, U. S. Army.

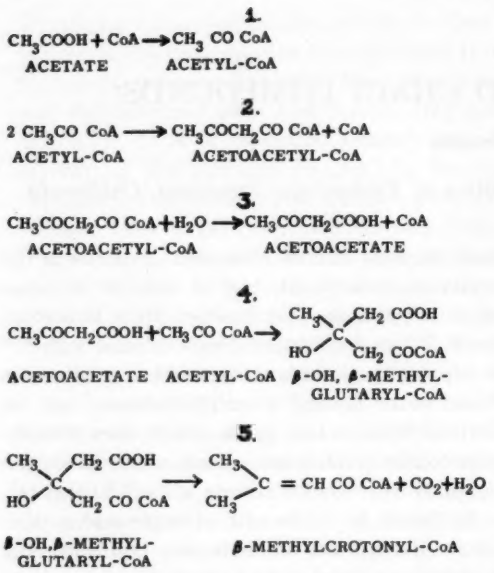


FIG. 1. Pathway of formation of isoprenoid intermediates in a plant enzyme system.

mitochondria of the flax seedling (5). Carboxyl-labeled acetate was incubated with the enzyme preparation and the products isolated by silicic acid column chromatography and two-dimensional paper chromatography. Acetate was found to be incorporated into acetoacetate and into BOG. The reactions are dependent on ATP and CoA. *Reactions 1*, *2*, and *3* have been studied in several systems and are common to the fatty acid metabolism as well as to isoprenoid synthesis. *Reactions 4* and *5* can be studied by running them in the reverse direction, with labeled β-methylcrotonate or labeled CO_2 as the substrate. As indicated above, BOG-CoA is formed from acetate, but extensive experimentation shows that BOG is not activated by spinach preparations or by whole bean leaves, nor is BOG degraded by these preparations. On the other hand, β-methylcrotonate is activated and transformed as is illustrated by the fact that β-methylcrotonate supports rubber formation in the intact plant and can be converted to BOG as well as to acetyl-CoA and acetoacetate in our flax seedling enzyme preparation.

Let us now turn to the possible role of β-methylcrotonyl-CoA as the monomer of terpene synthesis. How are two β-methylcrotonyl-CoA units put together to make a simple terpene if this is indeed the reaction? We do not know. We do know that β-methylcrotonate is rapidly metabolized to low molecular weight compounds by the guayule plant. These compounds, probably acidic in nature, appear to constitute a complex mixture of unsaturated fatty acids, as has been shown in our laboratory by Walter McNutt. It is possible that they may constitute intermediates in the formation of the longer chain isoprenoids from β-methylcrotonate itself. The fact that β-methylcrotonate is so rapidly and extensively metabolized by the plant into a variety of isoprenoid compounds of low molecular weight supports the idea that it is β-methylcrotonyl itself which is the unit condensed. Of this, however, we have as yet no direct evidence. Obviously, much remains to be done before the complete pathway of isoprenoid polymerization can be discussed in enzymatic detail.

REFERENCES

1. BONNER, J. AND A. W. GALSTON. *Bot. Rev.* 13: 543, 1947.
2. BONNER, J. AND B. ARREGUIN. *Arch. Biochem.* 21: 109, 1949.
3. BONNER, J. *J. Chem. Educ.* 26: 628, 1949.
4. ARREGUIN, B., J. BONNER AND B. J. WOOD. *Arch. Biochem.* 31: 234, 1951.
5. JOHNSTON, J. A., D. W. RACUSEN AND J. BONNER. *Proc. Nat. Acad. Sci.* 40: 11031, 1954.
6. BANDURSKI, R. S., T. COYLE AND J. BONNER. *Biology 1953*. Pasadena: Calif. Inst. of Technology, 1953.
7. ADAMS, R. AND B. L. VAN DUREN. *J. Am. Chem. Soc.* 75: 2377, 1953.
8. KLOSTERMAN, H. AND F. SMITH. *J. Am. Chem. Soc.* 76: 1229, 1954.

CONVERSION OF CHOLESTEROL TO BILE ACIDS[1]

MARVIN D. SIPERSTEIN AND I. L. CHAIKOFF

From the Laboratory of Chemical Pharmacology, National Heart Institute, National Institutes of Health, Bethesda, Maryland and the Department of Physiology, University of California School of Medicine, Berkeley, California

THIS discussion of the conversion of cholesterol to bile acids may be conveniently divided into two portions. We should like first to discuss the question of the quantitative importance of bile acids as end-products of cholesterol metabolism; and secondly, we shall deal with what is known about the intermediate compounds involved in the conversion of cholesterol to the bile acids.

BILE ACIDS AS END-PRODUCTS IN CHOLESTEROL METABOLISM

Our interest in these problems grew out of a project designed to reexamine, with the aid of carbon-14 labeling, the metabolic breakdown of cholesterol in the animal body. Although the subject of cholesterol catabolism has been extensively investigated over a period of years with the use of balance studies and with deuterium labeled cholesterol (1–4), it was felt that further information might be obtained by use of the more sensitive technique of radioactive carbon labeling. It might be well before discussing the cholesterol-C^{14} studies to mention some of the conclusions of these earlier experiments, many of which will bear directly on our findings. These can be briefly summarized as follows (fig. 1):

a) Cholesterol was believed to be converted either in the body or in the intestinal tract to two saturated, 27 carbon, neutral sterols; namely, dihydrocholesterol and coprosterol (5–8).[2] *b*) It was concluded that coprosterol was the chief end-product in the metabolism of cholesterol (10, 3, 4). *c*) Balance studies showed that if large amounts of cholesterol were fed, some of the cholesterol was converted to compounds which were no longer detectable by means of digitonin precipitation, thus giving some indication that cholesterol could be metabolized by routes other than those just mentioned (11–13). *d*) It was demonstrated by Bloch (14) and by Bloch, Berg and Rittenberg (15) with the use of deuterium labeled cholesterol, that both pregnanediol and cholic acid could be derived from cholesterol.

In reinvestigating this problem we were fortunate in being able to employ cholesterol labeled with carbon-14 in either of two positions (fig. 2). Cholesterol-4-C^{14} was synthesized by Dauben and Eastham (16), and cholesterol labeled in carbon-26, one of the terminal carbons of the side chain, was prepared by Dauben and Bradlow (17).

Our first experiments with the labeled cholesterol were designed to determine simply whether either of these tagged carbons could be oxidized in the body to carbon dioxide. Individual rats were given cholesterol-26-C^{14} or cholesterol-4-C^{14} intravenously as an emulsion with glycerol monostearate and triolein. Radioactive assay of the expired air indicated that whereas carbon-4 of cholesterol is not oxidized to carbon dioxide in the body, the terminal carbons of the isooctyl side chain are extensively excreted as carbon dioxide (18).

In the five rats receiving cholesterol-26-C^{14}, an average of 19% of the injected cholesterol molecules lost at least one of their terminal carbons in the course of 24 hours. If the period of collection was continued for 84 hours, as much as 40% of the 26 label could be found in the expired air. No $C^{14}O_2$ was excreted in the 84-hour period by the rats receiving cholesterol-4-C^{14} (fig. 3).

This finding that a large portion of the body's cholesterol could be partially degraded to carbon dioxide seemed at variance with the earlier conclusions that 27-carbon compounds constitute the chief end-products of cholesterol metabolism. An alternative explanation was, however, remotely possible; that is, the labeled terminal carbons could be turning over at a faster rate than the remainder of the cholesterol molecule, thus yielding $C^{14}O_2$ and still preserving a 27-carbon structure. The extensive loss of the labeled carbons from the sterol side chain did, nonethe-

[1] The studies reported here were in part supported by grants from the Public Health Service and the Life Insurance Medical Research Fund.

[2] The place of cholestenone as an intermediate in this reaction is now seriously in doubt (9).

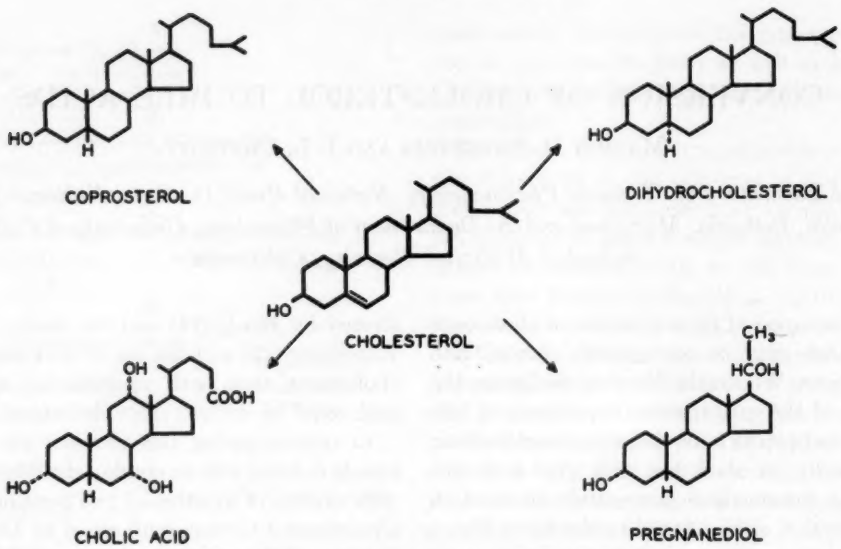


FIG. 1. Known interconversions of cholesterol.

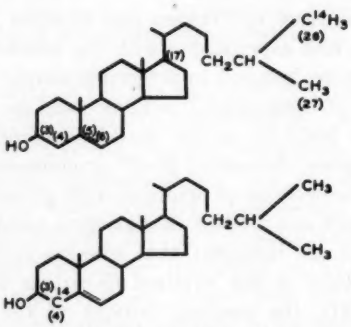


FIG. 2. Sites of C^{14} labels.

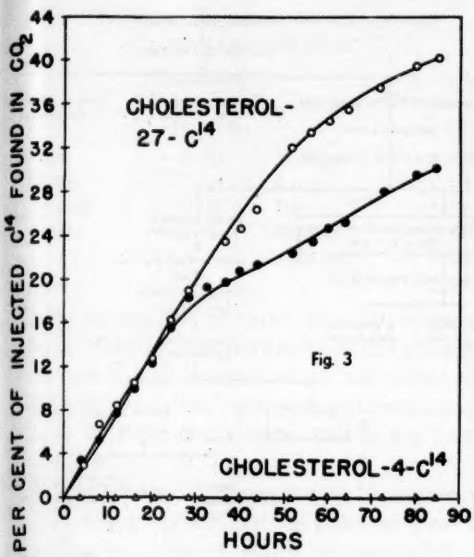
less, lead us to suspect that a sterol with fewer than 27 carbons, such as a bile acid, might play a more important part in cholesterol catabolism than had been previously thought.

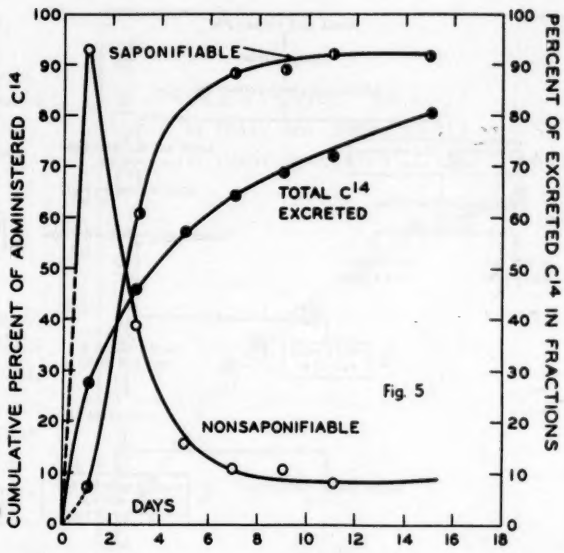
Further studies were then carried out on the excretion of compounds containing carbon-4 of cholesterol (19). It was found that whether cholesterol was injected as an emulsion or as a Tween solution or if it was given by mouth, the end-products of cholesterol metabolism which contained this label were eliminated almost entirely by way of feces.

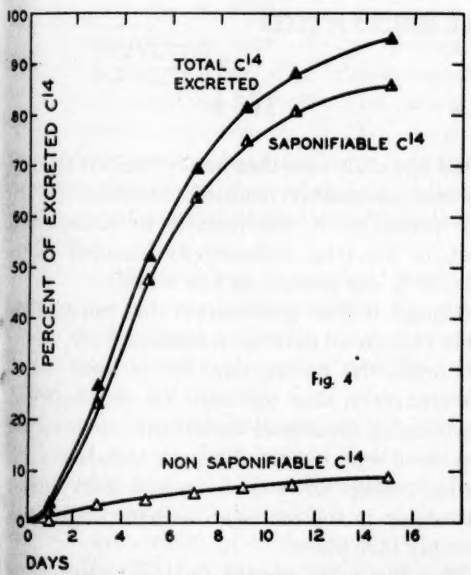
When cholesterol-4-C^{14} was administered intravenously a comparison of the various routes of excretion of carbon-4 indicated that less than 1% was eliminated in urine and, as already noted, none appeared in the expired air. On the other hand, if rats were maintained for a period of 15 days, as much as 95% of the injected C^{14} could be recovered in the feces (fig. 4).

The question then arose as to whether the major fecal end-product of cholesterol metabolism was coprosterol or whether the loss of carbon-26 as CO_2 did indicate that smaller compounds were responsible for the fecal C^{14}. Some idea as to the nature of these fecal excretion products could be obtained by simply saponifying the feces and separating the radioactivity into two fractions: the nonsaponifiable fraction which would include cholesterol itself, dihydrocholesterol and coprosterol; and the saponifiable fraction which includes both bile acids and fatty acids. As will be noted, when this was done less than 10% of the activity could be recovered in the nonsaponifiable portion of the feces, clearly indicating that coprosterol is not an important end-product in cholesterol catabolism.

The criticism could be made of the previous experiment that the results might reflect an abnormal metabolism of cholesterol caused by administering the compound by the intravenous route. To eliminate this possibility a similar study was performed in a rat which received cholesterol-4-C^{14} by stomach tube. As can be seen in figure 5, in a 15-day period 81% of the C^{14} of the administered cholesterol had been eliminated in the feces. The 30% of the C^{14} which appeared during the first day or two was primarily in the form of non-saponifiable material and no doubt represents cholesterol which was never absorbed from the intestine. However, after this initial period the fraction of C^{14} in the nonsaponifiable portion decreased rapidly and averaged only about 11% during the remainder of the study. This experiment with orally administered cholesterol-C^{14} again indicated that it was in the







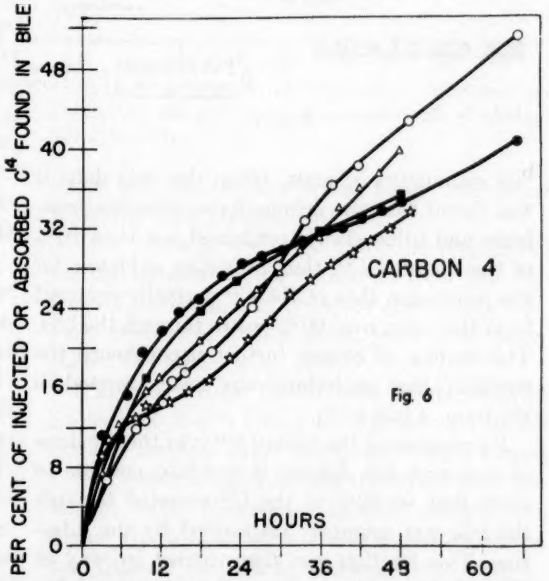


FIG. 3. Excretion of $C^{14}O_2$ in normal rats receiving either cholesterol-4-C^{14} or cholesterol-26-C^{14} (carbons-26 and 27 of cholesterol are equivalent).

FIG. 4. Excretion of C^{14} in feces following intravenous administration of cholesterol-4-C^{14}.

FIG. 5. Excretion of C^{14} in feces following oral administration of cholesterol-4-C^{14}.

FIG. 6. Biliary excretion of C^{14} following administration of cholesterol-4-C^{14}. *Solid symbols* represent rats receiving cholesterol by mouth; *open symbols* denote those receiving cholesterol intravenously.

saponifiable fraction that the metabolic products of the body's cholesterol were to be found.

To determine whether the labeled nucleus carbon of cholesterol was entering the intestinal tract by way of bile, cholesterol-4-C^{14} was next injected or fed to rats which were previously provided with bile fistulae. It was soon clear that carbon-4 of cholesterol was very rapidly excreted from the body by way of the bile. An average of 38% of the administered C^{14} was found in the collected bile in a 48-hour period. Again, neither route of administration nor type of preparation made any detectable difference in this rate of excretion (fig. 6).

Although the large amounts of C^{14} appearing in bile indicated that this route must be an important one in cholesterol excretion, the quantitative significance of this pathway could be estimated only by observing the amounts of C^{14} being excreted in the feces and urine of these

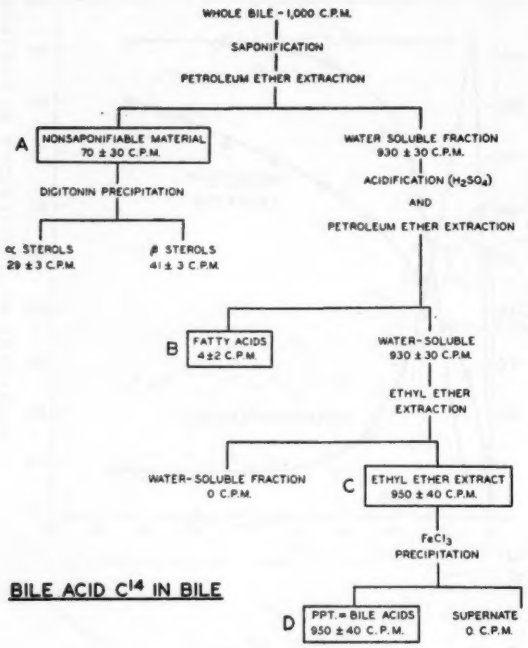


FIG. 7

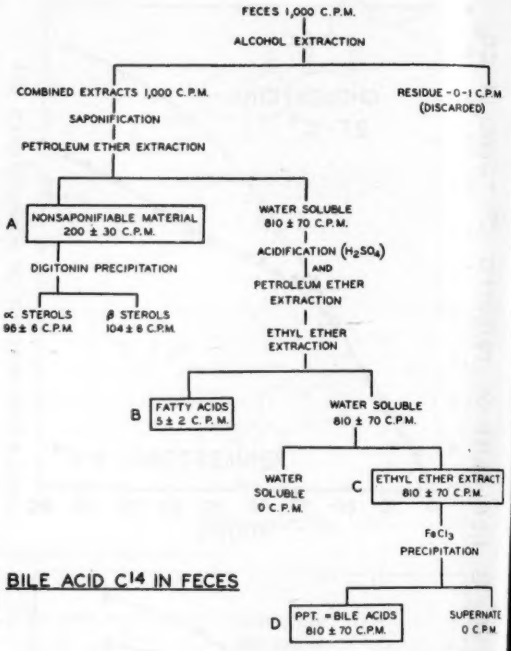


FIG. 8

bile cannulated animals. When this was done it was found that the acholic feces, intestinal contents and urine always contained less than 10% of the excreted C^{14}, thus providing evidence for the conclusion that of the C^{14} normally excreted from the body, over 90% passes through the bile. This finding, of course, further strengthened the suspicion that cholesterol was being excreted in the form of bile acids.

By re-infusing the biliary C^{14} into the duodena of rats with bile fistulae it was also possible to show that 80–90% of the C^{14} excreted through the bile was promptly reabsorbed by the intestine. This fraction was then carried by way of the portal blood to the liver, where it was quickly re-excreted into the bile (20). This enterohepatic circulation in an intact animal would allow only a small portion of the biliary C^{14} to escape into the feces with each cycle, but it is apparent that it was the sum of these fractions that in the intact rat led to the almost complete excretion of the injected cholesterol-4-C^{14} into the feces in a 15-day period.

Further chemical analyses of both bile and feces collected in the previous experiments were next undertaken (fig. 7, ref. 21). Total bile acid activity was obtained by first extracting the acidified, saponifiable fraction with petroleum ether to remove fatty acids. Following this, bile acids could be extracted with little or no contaminating radioactivity by shaking with ethyl ether. Total bile acids were then finally purified through the iron precipitation method of Doubilet (22). By this procedure it was possible to demonstrate that, of the total radioactivity excreted in the bile, 95% was present as bile acids.

Though it does demonstrate that most of the body cholesterol must be metabolized by way of bile acids, this finding does not of itself permit the conclusion that bile acids are the final end-products of cholesterol metabolism. During the process of repeated enterohepatic circulation and during passage down the intestinal tract further alterations in the bile acid molecule might conceivably take place.

That bile acids are the final end-products of cholesterol metabolism is demonstrated by the results obtained by analyzing the feces of the intact rat (fig. 8). In these experiments as much as 95% of the radioactivity of the administered cholesterol-C^{14} could be recovered in the feces, and of this amount over 80% was found to be present in bile acids (21).

From these experiments, therefore, it seems possible to conclude that only about 20% of the body's cholesterol is excreted in the feces as neutral sterols, i.e., without loss of any carbons. These arrive in part through the intestinal wall and in part via the bile.

On the other hand, the catabolism of over 80% of the body's cholesterol does involve the loss of the terminal three carbons of the cholesterol side

TABLE 1. IN VITRO OXIDATION OF CHOLESTEROL-26-C^{14}

Tissue	% Added C^{26} Oxidized to CO_2	Tissue	% Added C^{26} Oxidized to CO_2
Liver	0.30	Spleen	0.03
Kidney	0.12	Lung	0.01
Adrenal	0.08	Brain	0.01
Testes	0.07	Boiled Liver	0.00
			0.01

chain, at least one of which promptly appears as carbon dioxide. The remainder of the cholesterol molecule is then excreted in the bile as bile acids, which after repeated enterohepatic circulation are finally excreted in the feces, still in the form of bile acids.

While this work was appearing Bergström provided strong support for one phase of these conclusions in a report which demonstrated that cholic acid, the chief bile acid excreted in rat bile, accounted for most of the radioactivity which he found in bile after intraperitoneal injection of cholesterol-4-C^{14} (23). This finding was subsequently confirmed by us (24). Bergström and later ourselves have further shown that it is as the taurine conjugate of cholic acid that the rat excreted most of its cholesterol into the bile (24, 25).[3]

Although the work so far described was carried out in the rat, some evidence that the mechanism of cholesterol metabolism just discussed is not confined to this species was provided by the observations that the dog can oxidize carbon-26 but not carbon-4 of cholesterol to CO_2 (19), and can also convert cholesterol to cholic acid (15).

Recently at the National Institutes of Health we have been able to extend this work by studying the metabolism of cholesterol-4-C^{14} in a human being (27). In a man provided with a biliary fistula, a total of about 40% of an intravenous dose of cholesterol was excreted from the body in a 50-hour period. Of this amount 98.5% was found in the bile, whereas only 1.3 and 0.2% were excreted in the acholic feces and urine respectively. No $C^{14}O_2$ was detected in the expired air. Fractionation of the biliary C^{14} revealed that in man, too, approximately 90% of the activity excreted in bile is present as bile acids. By isotope dilution and paper chromatography the major C^{14}-containing bile acid was again identified as

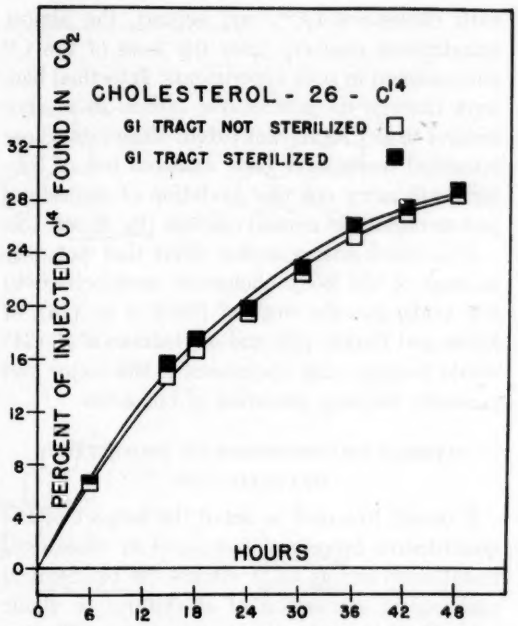


FIG. 9. Influence of intestinal bacteria on ability of the rat to oxidize carbon-26 of cholesterol to CO_2.

cholic acid, but in man it was found conjugated primarily with glycine.

The organs of the body which are responsible for the removal of the carbon-26 from the cholesterol molecule, and therefore presumably convert cholesterol to bile acids, have been determined by Meier *et al.* (28).

Slices of various tissues were incubated with cholesterol-26-C^{14}; CO_2 was collected and its C^{14} content assayed. Liver was found to be the most active organ in carrying out the removal of carbon-26 from cholesterol; kidney, adrenal and testes were less so, while the other organs tested had little or no activity (table 1).

It is interesting that this oxidation of carbon-26 has been further localized to the mitochondria of the liver cell by the work of Anfinsen, Horning, and Fredrickson (29, 30). Recently it has also been shown that mitochondria of liver will carry out the conversion of cholesterol-4-C^{14} to bile acids as isolated by the Doubilet procedure (22).

The question of the importance of intestinal bacteria in the oxidation of cholesterol also deserves consideration, especially since Dr. Thressa Stadtman has found that some species of bacteria will oxidize carbon-4 of cholesterol to CO_2 at a more rapid rate than carbon-26 (32). That intestinal bacteria do not carry out such a reaction is amply demonstrated by, first, the lack of $C^{14}O_2$ recovered from the intact rat or man injected

[3] The isolation of cholyl CoA and the demonstration of its role as an intermediate in taurocholic acid synthesis has been reported at these meetings (26).

with cholesterol-4-C^{14}, and second, the almost quantitative recovery from the feces of the C^{14} administered in such experiments. Intestinal bacteria likewise do not remove carbon-26 of cholesterol to any significant extent since rats whose intestinal tracts have been rendered free of bacteria will carry out this oxidation of cholesterol just as rapidly as normal animals (fig. 9, ref. 18).

It is worth noting at this point that not only is most of the body cholesterol metabolized to bile acids, but the work of Bloch *et al.* (15), of Zabin and Barker (33) and of Hellman *et al.* (34) would indicate that cholesterol is the major and probably the only precursor of bile acids.

PATHWAY OF CONVERSION OF CHOLESTEROL TO CHOLIC ACID

I should like now to leave the subject of the quantitative aspects of bile acids in cholesterol metabolism and go on to discuss the biochemical pathway of conversion of cholesterol to cholic acid. Unfortunately this pathway is still very poorly understood.

When the labeled bile acids of rats injected with cholesterol-4-C^{14} were first separated by paper chromatography, it was apparent that at the early intervals of bile collection most of the radioactivity was found not in cholic acid but in another unidentified bile acid. It was only several hours after the cholesterol injection that, simultaneously with a decrease in radioactivity in this unknown acid, C^{14} began to appear in cholic acid (24).

This suggested to us the possibility that the compound in question was a bile acid intermediate between cholesterol and cholic acid. The unknown band gave color reactions and was located on paper chromatograms at a site very close to that of desoxycholic acid. However, desoxycholic acid isolated from bile after the addition of carrier failed to contain any radioactivity. Bergström has shown that this dihydroxylated bile acid is actually chenodesoxycholic acid, having hydroxyl groups on carbons 3 and 7 (25). This observation, together with the finding of Harold that the monohydroxylated bile acid, lithocholic acid, contains C^{14} soon after cholesterol-4-C^{14} injection led us to the idea that lithocholic could be converted to chenodesoxycholic acid which in turn would be hydroxylated to give cholic acid (fig. 10, ref. 24).

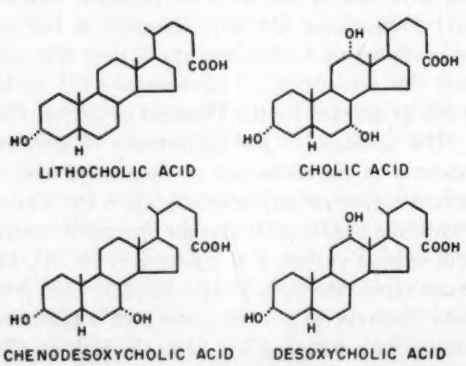


FIG. 10. Formulas of the commoner bile acids.

Bergström, however, has presented evidence that the last step in this sequence of reactions is not correct. Using C^{14} labeled lithocholic acid, he found that instead of forming cholic acid, lithocholic acid is converted in the rat to a compound which behaves on reverse phase chromatography as a trihydroxy bile acid, but which is definitely not cholic acid (35). This compound has not as yet been further characterized.

Bergström has provided further evidence on the role of the dihydroxylated bile acids in the conversion of cholesterol to cholic acid (36, 37). Desoxycholic acid-24-C^{14} and chenodesoxycholic acid-24-C^{14} were injected into rats and their metabolism observed. He noted that, whereas desoxycholic acid does yield cholic acid-C^{14}, chenodesoxycholic is converted at a slower rate to the unknown trihydroxy bile acid, which had also been formed from lithocholic acid.

If desoxycholic acid is an intermediate in the conversion of cholesterol to cholic acid in the rat, the conversion of C^{14}-cholesterol to desoxycholic acid should be demonstrable. Until recently desoxycholate had not been shown to be present in rat bile, and after injecting C^{14}-cholesterol, the isotope dilution method with pure desoxycholic acid had not demonstrated C^{14} to be present in this compound. In the past few months, however, use of the trapping technique has enabled us to show that desoxycholic acid can be identified in the rat and further that cholesterol-C^{14} is converted to it at an appreciable rate. Thus, after

TABLE 2. CHOLESTEROL-C^{14} CONVERSION TO DESOXYCHOLATE-C^{14}

Crystallization	Desoxycholate Specific Activity, (cts/min/mg)
1	3.2
2	3.2
3	3.0
4	3.2

Bile fistula rat injected with 100 γ of cholesterol-4-C^{14} and 20 mg of desoxycholic acid. The biliary desoxycholic acid-C^{14} isolated with the aid of pure carrier had the above specific activities which are equivalent to a minimum of 9.8% of the excreted cholesterol-4-C^{14}.

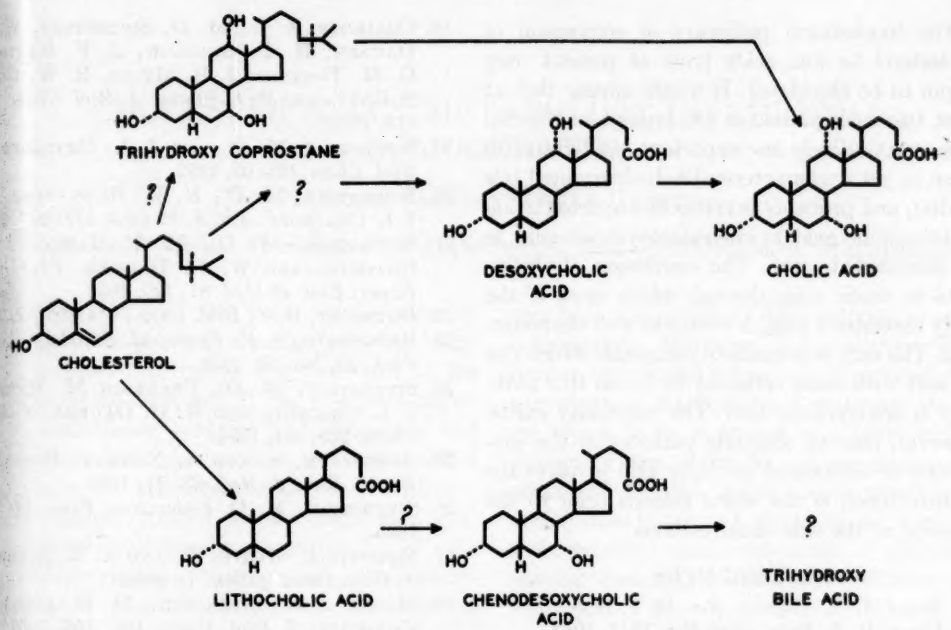


FIG. 11. Probable pathways of bile acid synthesis.

the injection of 20 milligrams of desoxycholate, approximately 10% of the cholesterol-C^{14} excreted during the next 6 hours could be recovered as this bile acid (table 2, ref. 38).

At our present state of knowledge, therefore, there is reason to believe that cholesterol can, in the rat, follow at least two pathways of breakdown to bile acids (fig. 11). One of these would involve the conversion of cholesterol to lithocholic acid, which in turn would be hydroxylated at the position to yield chenodesoxycholic acid. This compound on hydroxylation may then become the uncharacterized trihydroxylated bile acid.

A second and quantitatively more important pathway would consist of the conversion of cholesterol to desoxycholic acid, which would then be rapidly hydroxylated in the 7 position to give cholic acid as a final end-product.

The intermediate steps in the conversion of cholesterol to either lithocholic acid or to desoxycholic acid have not as yet been described. The work of Zabin and Barker, however, has demonstrated that this conversion involves the loss of only the isopropyl carbons of the cholesterol. In other words, in this process the side chain of cholesterol is not extensively broken down to be later resynthesized to that of cholic acid (33).

There is some indication that in the conversion of cholesterol to cholic acid, hydroxylation of the nucleus of the neutral sterol may precede the oxidation of the side chain. Bergström (39) has prepared 3,7,12 trihydroxy coprostane and has shown that this compound is converted in the body to cholic acid at a rapid rate. If this sterol is actually on the natural pathway of conversion of cholesterol to cholic acid it might indicate the presence of a third route by which bile acids are formed from cholesterol.

Finally, I should like to mention the question of reversibility of the above reactions. Byers and Biggs have shown that tritium labeled cholic acid is not converted to cholesterol by the rat (40) and Bergström has reported that cholic acid-24-C^{14} is not changed into any less hydroxylated bile acids (36). Likewise, little if any less polar compounds are formed in the rat from chenodesoxycholic-C^{14} (37). The evidence would indicate, therefore, that the conversion of cholesterol to cholic acid in the intact animal progresses in only the one direction.

SUMMARY

In summary, then, it can be said that studies in the rat have demonstrated that the chief pathway of catabolism of cholesterol involves its oxidation to bile acids, and it is as bile acids that cholesterol is eliminated both in the bile and in the feces. The importance of these conversions in human cholesterol metabolism has also been established. In rat bile the major bile acid end-product of cholesterol metabolism has been identified as taurocholic acid, while in human bile cholesterol is eliminated primarily as glycocholic acid.

The biochemical pathways of conversion of cholesterol to bile acids have at present only begun to be elucidated. It would appear that at least two such pathways are present in the rat. The quantitatively less important one terminates in an as yet uncharacterized trihydroxylated bile acid(s), and probably involves the hydroxylation of lithocholic acid to chenodesoxycholic acid as an intermediate step. The conversion of cholesterol to cholic acid, through which most of the body cholesterol goes, is even less well characterized. The only intermediate compound which can be said with some certainty to be on this pathway is desoxycholic acid. The possibility exists, however, that an alternate pathway in the conversion of cholesterol to cholic acid involves the hydroxylation of the sterol nucleus prior to the removal of the side chain carbons.

REFERENCES

1. Bills, C. E. *Physiol. Rev.* 15: 1, 1935.
2. Cook, R. P. *Nutr. Abst. Rev.* 12: 1, 1942.
3. Weinhouse, S. *Arch. Path.* 35: 438, 1943.
4. Bloch, K. *Circulation* 1: 214, 1950.
5. Rosenheim, O. and T. A. Webster. *Nature* 136: 474, 1935.
6. Rosenheim, O. and T. A. Webster. *Biochem. J.* 37: 513, 1943.
7. Schoenheimer, R., D. Rittenberg and M. Graff. *J. Biol. Chem.* 111: 183, 1935.
8. Anker, H. S. and K. Bloch. *J. Biol. Chem.* 178: 971, 1949.
9. Rosenfeld, R. S., David K. Fukushima, Leon Hellman and T. F. Gallagher. *J. Biol. Chem.* 211: 301, 1954.
10. Dam, H. *Biochem. J.* 28: 815, 1934.
11. Page, I. H. and W. Menschick. *J. Biol. Chem.* 97: 359, 1932.
12. Menschick, W. and I. H. Page. *Ztschr. Physiol. Chem.* 218: 95, 1933.
13. Schoenheimer, R. and F. Bruesch. *J. Biol. Chem.* 103: 439, 1933.
14. Bloch, K. *J. Biol. Chem.* 157: 661, 1945.
15. Bloch, K., B. N. Berg and D. Rittenberg. *J. Biol. Chem.* 149: 511, 1943.
16. Dauben, W. G. and J. F. Eastham. *J. Am. Chem. Soc.* 73: 3260, 1951.
17. Dauben, W. G. and H. L. Bradlow. *J. Am. Chem. Soc.* 72: 4248, 1950.
18. Chaikoff, I. L., M. D. Siperstein, W. G. Dauben, H. L. Bradlow, J. F. Eastham, G. M. Tomkins, J. R. Meier, R. W. Chen, S. Hotta and P. A. Srere. *J. Biol. Chem.* 194: 413, 1952.
19. Siperstein, M. D. and I. L. Chaikoff. *J. Biol. Chem.* 198: 93, 1952.
20. Siperstein, M. D., H. H. Hernandez and I. L. Chaikoff. *Am. J. Physiol.* 171: 297, 1952.
21. Siperstein, M. D., M. E. Jayko, I. L. Chaikoff and W. G. Dauben. *Proc. Soc. Exper. Biol. & Med.* 81: 720, 1952.
22. Doubilet, H. *J. Biol. Chem.* 114: 289, 1936.
23. Bergström, S. *K. Fysiograf. Sällskap. Lund, Förh.* 22, No. 16, 1952.
24. Siperstein, M. D., Franklin M. Harold, I. L. Chaikoff and W. G. Dauben. *J. Biol. Chem.* 210: 181, 1954.
25. Bergström, S. and A. Norman. *Proc. Soc. Exper. Biol. & Med.* 83: 71, 1953.
26. Siperstein, M. D. *Federation Proc.* 14: 282, 1955.
27. Siperstein, Marvin D. and A. W. Murray. *J. Clin. Investigation.* In press.
28. Meier, J. R., Siperstein, M. D. and I. L. Chaikoff. *J. Biol. Chem.* 198: 105, 1952.
29. Anfinsen, C. B. and M. G. Horning. *J. Am. Chem. Soc.* 75: 1511, 1953.
30. Fredrickson, D. S., M. G. Horning and C. B. Anfinsen. *Federation Proc.* 13: 212, 1954.
31. Fredrickson, Donald S. Personal communication.
32. Stadtman, T. C., A. Cherkes and C. B. Anfinsen. *J. Biol. Chem.* 206: 511, 1954.
33. Zabin, I. and W. F. Barker. *J. Biol. Chem.* 205: 633, 1953.
34. Hellman, L., R. Rosenfield, D. Fukushima, M. Eidinoff, A. Brunschwig and T. Gallagher. *J. Clin. Investigation* 32: 573, 1953.
35. Bergström, S., J. Sjövall and J. Volti. *Acta physiol. scand.* 30: 22, 1953.
36. Bergström, S., M. Rottenberg and J. Sjövall. *Ztschr. f. physiol. Chem.* 295: 278, 1953.
37. Bergström, S. and J. Sjövall. *Acta chem. scand.* 8: 611, 1954.
38. Siperstein, M. D. and A. W. Murray. Unpublished observations.
39. Bergström, S., K. Pääbo and J. A. Rumpf. *Acta chem. scand.* 8: 1109, 1954.
40. Byers, S. O. and M. W. Biggs. *Arch. Biochem. Biophys.* 39: 301, 1952.

CHOLESTEROL-4-C^{14} METABOLISM IN ADRENAL HOMOGENATES[1]

NICHOLAS SABA[2] AND OSCAR HECHTER

From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

WITHIN the past few years considerable insight has been obtained concerning the biochemical processes involved in the biogenesis of the adrenocortical secretion. It has now been definitively established that: *a*) both cholesterol and acetate are basic precursors of the corticosteroid hormones, *b*) corticosterone and/or 17-hydroxycorticosterone (cortisol) are the major end products (stoichiometrically considered) of the corticosteroidogenic sequence and *c*) adrenocorticotrophin (ACTH) accelerates the rate of corticosteroidogenesis *in vitro* (1).

Our present picture of the probable sequences involved in the biosynthesis of corticosteroids is shown diagrammatically in figure 1. Those reactions which have been established unequivocally are shown with solid lines; those with dotted lines represent probable reactions which have not been shown definitively. In its essentials, this is the same reaction sequence which was presented in 1950 (2) modified in that most of the dotted lines presented then are now solid and new dotted lines have been added. The primary reactions of the sequence involved in the conversion of cholesterol to corticosterone and cortisol was devised entirely on the basis of studies involving the isolation and characterization of products resulting from the perfusion of various steroids through adrenal glands. This pointed the way for the search of simplified systems (cell-free homogenates, enzyme extracts, etc.) which might carry out one or another part of the reactions in the sequence. Within a few years all of the reactions from pregnenolone to corticosterone and/or cortisol were obtainable in extracts or homogenates (1, 3, 4); with our recent demonstration that adrenal homogenates convert cholesterol-4-C^{14} to radiopregnenolone (5), each of the reactions postulated in the 'cholesterol sequence' has now been unequivocally demonstrated in adrenal homogenates. Moreover, the available evidence from *in vivo* studies is basically in agreement with this phase of the postulated sequence, and thus we appear to have arrived at a fairly complete outline of the route by which cholesterol is converted to the 'major' corticosteroids. The dotted lines in figure 1 reveal, nevertheless, that there remain considerable areas of uncertainty. Among these may be listed the following problems:

a) The corticosteroidogenic activity of ACTH in the perfused bovine adrenal appears to be the resultant of the acceleration of a single step, cholesterol to pregnenolone (6), which appears to be the rate-determining step in the overall sequence whereby cholesterol is converted to the 'major corticoids.' However, the mode of ACTH action, if indeed operative at this step, is almost entirely obscure.

b) Evidence derived from bovine adrenal perfusion studies (6, 7), indicates the presence of an alternative pathway of corticosteroidogenesis from acetate, which does not involve cholesterol as an obligatory intermediary, and which is not stimulated by ACTH. There is, however, no definitive evidence for the existence of such an 'alternative' pathway. If existent, what are the possible intermediaries involved? Does one of these enter the 'cholesterol sequence' at the level of pregnenolone or progesterone, or is yet another pathway involved?

c) With the elucidation of the structure of aldosterone (8), it is necessary somehow to incorporate this highly potent mineralocorticoid into our picture of corticosteroid biosynthesis. The discovery of aldosterone, which is secreted by adrenals in trace amount relative to cortisol and/or corticosterone, is so recent that we do not even know whether aldosterone is derived from cholesterol. The limited *in vitro* data available concerning its biogenesis indicates that desoxycorticosterone (DOC) is converted to aldosterone, and may be an intermediary (9). *In vivo* studies

[1] The studies described were aided in part by Medical Research and Development Board, Office of Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-255.

[2] Fellow of Kellogg Foundation; on leave from the Ministry of Agriculture, Weybridge, England.

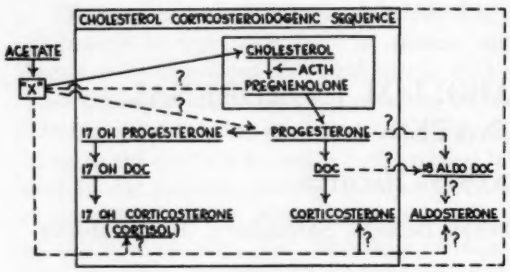


FIG. 1

strongly suggest, however, that ACTH exerts only minimal influence upon the rate of secretion of aldosterone, relative to its effect upon the adrenal output of corticosterone, cortisol, DOC, or 17-hydroxy DOC (10). The relative insensitivity of aldosterone production to ACTH is explicable on the basis that this corticoid does not arise primarily from the 'cholesterol sequence' which gives rise to corticosterone and cortisol, through DOC and 17 OH DOC respectively. Alternatively, aldosterone production could involve the 'cholesterol sequence' and still be relatively insensitive to ACTH, if an enzyme component of the aldosterone pathway had high affinity (or a preferential location) and a low saturation capacity for progesterone relative to the 17 and 21 hydroxylating enzyme systems. The primary position of progesterone hydroxylation (i.e. at 17, 21 or 18) might determine the subsequent fate of the progesterone molecule, whether to cortisol, corticosterone or aldosterone. Enzyme systems with similar characteristics have previously been postulated (11) to account for the production of certain trace products of progesterone metabolism by the perfused adrenal which are formed in constant amount independent of ACTH or progesterone concentration, in marked contrast to cortisol and corticosterone.

Our approach to the elucidation of these problems has involved search for a cell-free adrenal preparation which has the capacity of forming corticosteroids from both C^{14}-cholesterol and C^{14}-acetate. Given such a system, it might be possible to isolate intermediaries as well as end products, if the orderly sequence of reactions occurring within the intact cell could be disrupted and sufficiently disorganized. Examination of the relative specific activities of radioactive products formed, studied on a kinetic basis, should then provide evidence concerning the postulated 'non-cholesterol' pathway and its quantitative importance in corticosteroidogenesis under a variety of *in vitro* conditions. By suitable fractionation of the adrenal preparation it might be possible to study the step, cholesterol to pregnenolone, upon which ACTH appears to act, and thus gain some understanding of the mechanism of an *in vitro* hormone action. Finally, by incubating suitably labeled precursors, the biogenesis of aldosterone might be clarified.

The program outlined above (aside from the problem of aldosterone biogenesis) undertaken several years ago, was initiated by studying the metabolism of cholesterol-4-C^{14} in homogenates of cow adrenals. More recently, in conjunction with Drs. S. A. Simpson and J. F. Tait, we have been studying the biosynthesis of aldosterone in this system. It must be stated immediately, that the program undertaken has not been completed nor have we settled any of the specific problems we set out to elucidate. Instead, as the work progressed, we encountered one surprise after another with attendant violence to some of our preconceived notions. The work we present today must be considered as a progress report, wherein a few facts are combined with preliminary data to present a most incomplete picture.

PRODUCTS OF C^{14} CHOLESTEROL CATABOLISM IN ADRENAL HOMOGENATES

Preliminary work revealed that cell-free homogenates of freshly obtained cow adrenals prepared in a variety of media catabolized cholesterol-4-C^{14} to more polar products. Of the various homogenizing fluids tested, a hypotonic solution consisting of phosphate buffer (M/150, pH 7.4) supplemented with DPN, ATP, niacinamide, Mg, fumarate and sucrose was particularly useful, since such homogenates catabolized cholesterol in good yield, while permitting the accumulation of early intermediaries in the sequence, in amount sufficient for their ready detection by our technics. Table 1 illustrates the radioactive products obtainable from a representative experiment of this series, wherein 50 micrograms of cholesterol-4-C^{14} (2.5×10^5 ct/min.) was incubated with the total homogenate obtained from 100 grams of decapsulated-demedullated cow adrenals. The radioactive products were extracted from the incubation mixture with acetone and ethyl acetate; the crude lipoids thus obtained were first fractionated on silica gel, then by paper chromatography. All radioactive zones detectable by counting the paper strips were then further purified by repeated paper chromatography and fi-

nally silica gel chromatography in order to obtain crystalline products. Of the total count added, 14% was converted to a mixture of radioactive products. As table 1 reveals, four products could be identified by infrared (IR) spectroscopy: progesterone, pregnenolone, corticosterone and cortisol. Also present, but unidentified, were the following radioactive zones: *1*) 'A$_1$,' appears to be a single product of polarity comparable to 11-dehydrocorticosterone, which gives spot reactions characteristic for the Δ^4-3 ketone function, but not for the ketol side chain; *2*) 'E,' of polarity comparable to cortisone, consists of a mixture of Δ^4-3 ketones, one of which appears to be aldosterone and *3*) 'MPF,' a mixture of products more polar than cortisol. It has not been possible, as yet, to establish whether the aldosterone in zone 'E' is derived from the radiocholesterol.

The point of greatest interest to us was the finding that the early intermediaries of the sequence, pregnenolone and progesterone, had accumulated in the system in addition to the expected end products, cortisol and corticosterone. We have measured the specific activities of the four major catabolic products relative to the cholesterol isolated at the end of the experiment. The counts per mM per minute of the pregnenolone or progesterone is about 2.5 times greater than the isolated cholesterol, and about 60% greater than that of the isolated corticoids.

In the absence of kinetic data, the basis of the differences in specific activities between pregnenolone and progesterone on the one hand, and cortisol and corticosterone on the other cannot be definitively evaluated. However, the differences between the relative radioactivities of the cholesterol and the products isolated is so great, that it must be concluded that there are at least two pools of cholesterol in the total homogenate, only one of which is metabolically active in corticosteroidogenesis.

DISTRIBUTION OF CHOLESTEROL CATABOLIC ACTIVITY AND OF CHOLESTEROL IN SUB-FRACTIONS OF ADRENAL HOMOGENATES

The suggestion that there are different pools of cholesterol in the adrenal cortex made it necessary to determine the location of cholesterol in the cow adrenal cortical cell, and to determine whether the cholesterol catabolic activity was localized in a particular fraction of the homogenate. For these studies, glands were homogenized in a 'loose-fitting' Potter apparatus, with isotonic sucrose. The resulting homogenate was then fractionated by differential centrifugation, using the procedures of Schneider and Hogeboom (12).

TABLE 1. C^{14} PRODUCTS OF CHOLESTEROL METABOLISM IN TOTAL HOMOGENATE*

C^{14} Products†	Total Ct/ Min. × 10^3	Ct/ mM/ Min. × 10^5	% Total C^{14} Products
cholesterol		5.4	
progesterone‡	2.3	13.5	6.6
pregnenolone‡	3.5		10.0
'A$_1$'	2.5		7.1
corticosterone‡	17.8	8.3	50.0
'E'	2.5		7.1
cortisol‡	7.4	8.7	21.0
'MPF'	+§		1

* Adrenal cortex homogenate (100 gm) in 400 ml cofactor-buffer medium containing .025 sucrose incubated with 50 μg cholesterol-4-C^{14} (containing a total of 250 × 10^3 ct/min.) for 2.5 hr. at 38° (O_2).

† In order of increasing polarity.

‡ Identified by infrared analysis.

§ Detectable in trace amount.

To determine the site of cholesterol metabolism, the isolated washed fractions of the homogenate were suspended in the cofactor-phosphate buffer medium, and incubated with cholesterol-4-C^{14}. The radioactive products formed were measured by fractionating the lipoid extracts obtained, first on silica gel columns and then by paper chromatography using the propylene glycol-ligroin system, where cholesterol travels with the front. The cholesterol zone was located by color tests and count and the remaining portion of the papergram containing products more polar than cholesterol was eluted and counted to give an estimate of the radioactive products.

Upon testing the individual fractions of the homogenate separately, it was consistently observed that the 'mitochondrial' fraction (sedimented by 8,000 *g* for 20 min.) had most or almost all of the cholesterol-catabolic activity, whereas the 'nuclear' fraction (obtained by centrifugation at 600 *g* for 20 min.) and the combined supernate plus 'microsomes' were almost completely inactive.

In one such experiment, the isolated and washed fractions derived from 10 grams adrenal cortex were suspended in cofactor-phosphate buffer (M/150 phosphate pH 7.2, $MgSO_4$, fumarate, niacinamide, all at 5 × 10^{-3} M; ATP, 2.5 × 10^{-3} M;

TABLE 2. CHOLESTEROL DISTRIBUTION IN COW AND RAT ADRENAL FRACTIONS (MG CHOLESTEROL/100 GM ADRENAL)

	Free			Ester		
N*	M†	S‡	N*	M†	S‡	Total
Cow (1)						
13	64	94	3	4	29	207
Rat (2)						
105	224	640	144	286	1900	3290

* Nuclear fraction. †Mitochondrial fraction. ‡Supernate plus microsomes.

(1) Mean values of 4 cow glands
(2) Mean values of 16 rat glands

DPN, 5×10^{-4}M) and incubated at 38° for 2 hours in oxygen, with 5 micrograms cholesterol-4-C^{14} per 40 milliliters (containing a total of 25×10^3 ct/min.). The total counts per minute of the isolated products of the three fractions were: 'nuclear' fraction, 400; 'mitochondrial,' 2000; 'microsomal' plus supernate fraction, 50.

DISTRIBUTION OF CHOLESTEROL IN ADRENAL CELL FRACTIONS

Table 2 shows the distribution of cholesterol, both 'free' and esterified, in various fractions of cow adrenal homogenates; for comparison, results of similar fractionations of adrenals obtained from rats, hypophysectomized 24 hours previously, are also presented. In these experiments the cholesterol present in each fraction was separated into its 'free' and 'ester' forms by silica gel chromatography of the lipoid extracts of each fraction; after saponification, the cholesterol was separated as the digitonide and measured as the Lieberman-Burchard chromogen.

These data demonstrate that: *a*) cow adrenals obtained after slaughter contain very little cholesterol (most of which is unesterified) relative to the rat, where the bulk of the cholesterol is present in ester form. The significance of this difference between cow and rat adrenals has previously been discussed (13). *b*) In the cow gland the 'mitochondrial' fraction, the apparent locus of the cholesterol-catabolic system, contains a sizable proportion of the total cholesterol present in the cell and about 40% of the total 'free' cholesterol.

PRODUCTS OF C^{14} CHOLESTEROL CATABOLISM IN ADRENAL HOMOGENATES

The only enzyme in the course of reactions from pregnenolone to cortisol and corticosterone (cf. fig. 1) which is known to be associated with the adrenal mitochondria is the C-11 β hydroxylating system (1, 14) which is regarded as the terminal step in the sequence. The other enzymes involved, i.e. the 3β dehydrogenase (which converts pregnenolone to progesterone) as well as the C-17 and C-21 hydroxylating enzyme systems, are believed to be localized in the microsomes or in the supernatant fraction (1, 3). When we found that 'cholesterol-catabolic activity' was primarily associated with the mitochondria, our expectation was that the major product of C^{14} cholesterol metabolism by adrenal mitochondrial fractions should be pregnenolone if the other enzymes in the sequence (with the exception of the 11 hydroxylating system) were not present in mitochondria. If pregnenolone could be 11 hydroxylated by the mitochondrial enzyme, then 11β hydroxypregnenolone might be a product.

When the radioactive products obtainable from 'small-scale' mitochondrial incubations (derived from 4–6 gm adrenal cortex per incubation) were fractionated by paper chromatography, the results were most surprising. The major radioactive zone was of mobility equivalent to progesterone, a variety of radioactive zones more polar than progesterone (MPP) were observed, but despite careful search, no radioactive zone of mobility equivalent to pregnenolone could be detected.

Our inability to detect pregnenolone in mitochondrial incubations was difficult to explain in view of its clear accumulation in total adrenal homogenates, and raised the possibility that pregnenolone might not be an intermediary in the biogenesis of corticoids from cholesterol as we had postulated. Alternatively, it is possible that there might be factors present in total homogenates which influence the rates of reaction in such a way that pregnenolone accumulates in that system. Accordingly, we undertook investigations designed to test some factors, present in the total homogenate, which might influence cholesterol metabolism in mitochondria and lead to the accumulation of pregnenolone.

Mitochondria incubated in total homogenates differ from those incubated in cofactor medium alone in at least four respects: *a*) the presence of non-mitochondrial cellular components, e.g. nuclei, microsomes and soluble protein factors present in the supernate; *b*) in Na/K ratio, since the cofactor medium was primarily Na, and the adrenal tissue contributed sizable K to the total homogenate; *c*) in ascorbic acid content, since total homogenates of cow adrenals contain about 235 milligrams ascorbate per 100 grams tissue, whereas the isolated mitochondria from this

amount of tissue contain only 12 milligrams; *d*) finally, in the total molarity of the medium, wherein the tissue contributes a number of osmotically active substances to the hypotonic cofactor medium.

Although our studies on the possible influence of the above factors are incomplete, particularly with respect to the possible interactions between non-mitochondrial structures and mitochondria, we have been unable, so far, to modify cholesterol metabolism in isolated mitochondria so that pregnenolone accumulates in detectable amounts.

If pregnenolone is not detectable, what are the products formed by mitochondrial action upon cholesterol-4-C^{14}? To answer this question we pooled the C^{14} products from 28 'small-scale' mitochondrial incubations (wherein we had studied factors like Na/K ratio, molarity, and ascorbic acid) and fractionated this mixture by systematic paper chromatography. As homogeneous zones were obtained, the radioactive material was then further fractionated on silica gel columns to remove impurities derived from the paper.

From a total of 10^6 counts per minute of C^{14} products, the major fraction (70%) was of polarity equivalent to progesterone, and following successive rechromatography on paper and silica gel, it was possible to isolate crystalline progesterone, which was definitely identified by infrared spectroscopy. The more polar products turned out to be a mixture of unknowns which we have not been able to identify. Of these, the major radioactive fraction is an unknown which we have designated as B_1; on repeated paper chromatography in propylene glycol-toluene it behaves as a homogeneous zone; in this system it has polarity similar to corticosterone. Appropriate spot tests reveal that B_1 shows evidence for a ketol side chain and a Δ^4-3 ketone function. B_1 can be differentiated from corticosterone and 17 hydroxy DOC, both by infrared (IR) analysis and by its failure to give a H_2SO_4 reaction on paper. The IR analysis reveals 3 carbonyl bands. If B_1 is a single compound, one carbonyl may be associated with the α-β unsaturated ketone system, one could be placed at C-20 to account for the ketol side chain, but we do not know where to place the third carbonyl function. At a time when everyone is 'aldosterone-conscious' it may be tempting to place the carbonyl at C-18, but we have no evidence for this. The second unknown, present in smaller amount than B_1, is a radiofraction, which on repeated chromatography in propylene glycoltoluene shows only one radioactive area. This is of polarity similar to 11-dehydrocorticosterone, and designated by us as A_2. A_2 gives spot tests for Δ^4-3 ketone but not for a ketol side chain; IR analysis reveals the same three carbonyl bands found in B_1. In addition to B_1 and A_2, traces of other unidentified products, more polar than cortisol and of polarity equivalent to cortisone, were observed.

Of major interest to us was the fact that despite a careful search, no trace of 11 β-hydroxyprogesterone, 17 α-hydroxyprogesterone, DOC, corticosterone or cortisol could be detected. We have already commented upon the fact that pregnenolone could not be detected in our preliminary surveys; it likewise could not be detected in the pooled sample. Apparently the mitochondria contained the enzymatic apparatus necessary to convert cholesterol to progesterone, but did not possess C-17, C-21, or C-11β hydroxylating activity at an effective site of the structure to permit further enzymatic transformation of the progesterone produced to produce cortisol or corticosterone. This is not to say that a C-21 hydroxylating system is not present, for it will be recalled that B_1 appears to be a ketol. In the absence of the above hydroxylating activities a new pathway of progesterone metabolism seemed to be operative in mitochondria to produce unidentified more polar products at a slow rate relative to the conversion of cholesterol to progesterone.

The nature of these apparently new C^{14} products was of sufficient interest to warrant their further investigation. To obtain sufficient mate-

TABLE 3. C^{14} PRODUCTS ISOLATED FROM 'LARGE-SCALE' MITOCHONDRIAL INCUBATIONS*

C^{14} Products	Total Ct/Min. $\times 10^3$	Ct/mM/ Min. $\times 10^3$	% Total C^{14} Products
cholesterol		14.3	
progesterone†	12.6	13.0	40
11β hydroxyprogesterone†	7.7		20
corticosterone†	5.2	8.9	16
'E'	3.0		10
cortisol†	3.1	9.2	10
'MPF'	+		

* Mitochondrial fraction obtained from an equivalent of 100 gm adrenal cortex, suspended in 400 ml of cofactor-buffer medium, containing 0.025 M sucrose, incubated with 50 μg. cholesterol-4-C^{14} (250 $\times$ 10^3 ct/min.) for 2.5 hrs at 38° (O_2).

† Identified by infrared analysis.

rial for identification purposes we decided to prepare mitochondrial fractions on a 'large-scale,' using the equivalent to 200–300 grams adrenal cortex, instead of the usual 'small-scale' preparation involving 50 grams of cortical tissue. Three experiments with mitochondria produced on a 'large scale' were performed and the products fractionated by paper chromatography. The major products were isolated and identified by IR analysis. The results of all experiments were essentially similar, but the products formed were completely different from those of the 'small-scale' mitochondrial preparations. We had undertaken the 'large-scale' experiments hoping to isolate and identify B_1 and A_2; neither of these could be detected. Instead, the products obtainable from 'large-scale' preparations shown in table 3 are very similar to those of total homogenates in that corticosterone, cortisol, and progesterone are major C^{14} products. An 'E' zone containing aldosterone as one component was also observed, as determined by bioassay and chromatography by Drs. Simpson and Tait but as with the homogenate experiments, it cannot be concluded that the aldosterone was radioactive although it was produced in the mitochondrial system.

The only qualitative differences between these mitochondrial preparations and total homogenates were the presence of 11β hydroxyprogesterone in the former, but not the latter, and the absence of pregnenolone as a mitochondrial metabolite of cholesterol. If the qualitative differences are small, Table 4 reveals that there are considerable quantitative differences between these preparations. Whereas cortisol and corticosterone constitute about 70% of the products in total homogenates, they account for about 26% of the C^{14} products in the 'large-scale' mitochondrial incubations. It is clear in these mitochondria experiments, that although the entire battery of enzymes involved in the conversion of cholesterol to corticoids is present, the rate of progesterone conversion to corticoids is slow compared to its formation from cholesterol in contrast to total homogenates. The presence of 11β hydroxyprogesterone as a product in these mitochondrial incubations, but not in homogenates, is probably the resultant of the accumulation of progesterone as a consequence of the limited capacity of the C-17 and C-21 hydroxylation activities to transform progesterone to cortisol and corticosterone.

TABLE 4. ACCUMULATION OF C^{14} PRODUCTS IN VARIOUS HOMOGENATE PREPARATIONS

Product	Total Homogenate	Mitochondrial Preparations	
		Large-scale	Small-scale
Progesterone	6.6	40	70
Pregnenolone	10.0	N.D.*	N.D.*
11β Hydroxyprogesterone	N.D.*	20	N.D.
Corticosterone	50.0	16	N.D.
Cortisol	21.0	10	N.D.

Values are % of total C^{14} products isolated.
* Not detectable.

What are the differences between the 'small-scale' and 'large-scale' experiments which could account for such a striking disparity? The question effectively resolves itself into a consideration of why apparently similar mitochondria exhibit C-17, C-21, and C-11β hydroxylating activities in one case (large-scale) but not in the other (small-scale). We have reviewed step by step all of our technics with respect to preparation of mitochondria, conditions of incubation, and isolation of products. The only difference which we think of which may explain these results involves a step in the spinning down of the mitochondria, after removal of nuclei and debris. In our 'large-scale' experiments it was necessary for us to spin down the mitochondria from 1500 milliliters of homogenate. We could centrifuge only 300 milliliters at a time in our centrifuge cups, and added additional homogenate to the mitochondrial pellet of the previous spinning. In our 'small-scale' experiments, where the total volume of homogenate was 300 milliliters, the mitochondria were spun down once and then resuspended for washing. The procedure employed in 'large-scale' workup concentrated the mitochondria some five times in the final spinning, and this may have resulted in the adsorption of non-mitochondrial enzymes which then could not be removed by two washings with isotonic sucrose.

Independent of the final explanation, these differences between 'large-scale' and 'small-scale' mitochondrial preparations point up the extreme difficulty in assigning particular enzyme activities to specific cellular structures. Subtle differences in technic produce marked qualitative differences. Thus it is not possible from these data to assign a mitochondrial location to some enzymes, but not others. All that can be said is that some enzyme activities are easier to dissociate

rom this organelle than others; in the case of the holesterol metabolic sequence, it is clear that the ystems involved in the conversion of cholesterol o progesterone are more firmly associated with itochondria than are the enzyme activities in-olved in the subsequent steps. Do results such s these signify that certain enzymes may be mobile' in the sense that they shift from one site n the cell to another, being temporarily adsorbed n the surface of a cell constituent and then re-eased? If so, this would provide a basis for ob-aining an orderly, directed series of reactions iving rise to specific end products.

In considering the 'cholesterol sequence' of igure 1, where some of the participating enzymes re considered to be non-mitochondrial, such a echanism involving adsorption of 'mobile' en-ymes on mitochondria would provide a basis for he orderly control of the cholesterol metabolic oute. Of the very large number of possible prod-cts which are formed in the conversion of choles-erol to corticoids, it should be recalled that even n the 'large-scale' mitochondrial incubations, nly a limited number actually accumulated. here is one further implication of the 'mobile nzyme' view which must be mentioned. The emonstration that a particular enzyme is pri-arily localized in a specific site in the cell need ear no necessary relationship to the cellular site t which the enzyme participates in a biochemical equence. For example, as a consequence of care-ul examination of cellular fractions of adrenal omogenates, Samuels and his associates (per-onal communication) have concluded that the 3 dehydrogenase activity which converts preg-enolone to progesterone is concentrated mainly n the 'microsomes;' small amounts of this enzyme re found in the mitochondrial fraction and this ctivity, while difficult to remove, can neverthe-ss be reduced by successive washing. On the ccepted view, the presence of this 'microsomal' nzyme in the mitochondrial fraction represents contaminant which arises because of the limita-ons of our present technics of fractionating cel-lar constituents. If, however, the mitochondrial dsorption thesis presented here be accepted, the lternative must be considered that only that ortion of the enzyme associated with the mito-ondria may be considered biochemically active terms of corticoid biogenesis; the bulk of the nzyme localized in microsomes may represent rely the storage site of activity which is poten-lly available.

SOME FACTORS WHICH INFLUENCE THE MITOCHONDRIAL METABOLISM OF CHOLESTEROL-4-C^{14}

During the course of these investigations, we had occasion to study the influence of a variety of factors upon the C^{14}-cholesterol catabolic activity of isolated mitochondrial fractions in 'small-scale' experiments. Although these studies are not complete, the following points seem worthy of note:

Ionic Composition. Alteration of the Na/K ratios in hypotonic media influences the percentage conversion of C^{14}-cholesterol to products. Using cofactor medium containing 0.025 M sucrose, the conversion in K is higher than in Na, but the ratio MPP/progesterone, determined by count, is unaltered.

Molarity. Addition of sucrose to the K-co factor medium to final concentrations up to 0.25 M (isotonic) had no striking influence on either the yield or the MPP/progesterone ratio of the C^{14} products.

Cofactor Requirements. Fumarate and Mg are necessary for maximal conversion of cholesterol to C^{14} products; these factors probably act at a stage prior to progesterone. ATP appears to have a 'restraining influence' on C^{14} cholesterol metabolism, exerted primarily at a level after progesterone; thus in its absence the MPP are increased 2–4 fold, while the accumulation of progesterone is diminished. DPN and niacinamide do not appear to be involved in the conversion of cholesterol to progesterone, although the MPP are decreased by the absence of DPN.

ACTH. We have tested the influence of ACTH upon mitochondrial metabolism of C^{14} cholesterol, under a variety of conditions. Under certain conditions we have obtained striking increases up to 200% in the rate of C^{14} cholesterol conversion by the addition of ACTH at a concentration of 1–2 IU per liter of medium. However, such effects have been obtained in only 2 of 5 trials and are not sufficiently consistent for us to conclude that ACTH acts directly on mitochondria.

Ascorbic Acid. It has been found that the addition of ascorbic acid (final concentration, 2.3×10^{-2} M) increases the percentage conversion of C^{14} cholesterol to radioactive products, and also increases the ratio of MPP/progesterone. This occurs both when hypotonic and isotonic media are used for suspending the mitochondria and is independent of the K/Na ratio.

CONCLUDING REMARKS

It is clear that these studies have answered none of the questions which prompted this investigation. Instead, a host of new questions have arisen to add further complexities to the problems of corticosteroid biogenesis. The role of pregnenolone as an intermediary in the conversion of cholesterol to progesterone in mitochondria now appears to be somewhat questionable. Although it is not possible from these data to exclude its role as an intermediary, it is difficult to account for our failure to detect pregnenolone in mitochondrial incubations, as opposed to its accumulation in total homogenates, on any simple kinetic basis. If pregnenolone were indeed a precursor of progesterone, under conditions where mitochondria markedly accumulate progesterone, one might expect that pregnenolone should also tend to accumulate. Studies with C^{14} labeled pregnenolone should clarify this difficulty.

The evidence which suggests a new pathway for cholesterol metabolism in mitochondria (small-scale) wherein a series of products are obtained in trace amount, focuses attention on an aspect of corticosteroidogenesis which has not received adequate attention. Finally, these studies have illustrated the complexity of the problem involved in localizing the specific enzymes concerned with cholesterol conversion to corticoids in terms of specific cell structures.

These difficulties which have arisen are to be welcomed, for they demonstrate that our initial picture of corticosteroid biogenesis, essentially primitive, is in a process of change and development. A mature picture of the biochemical processes involved in corticosteroid biogenesis will necessitate not only an understanding of a sequence in terms of individual chemical reactions, but an appreciation of the dynamic organizatio of the various components within the cell in reg ulating and directing these reations.

We wish to thank Mr. Paul Skogstrom for ad vice and interpretations concerning the infrare spectra, and Mr. Morris Feinstein for valuabl assistance.

REFERENCES

1. Hechter, O. and G. Pincus. *Physiol. Re* 34: 459, 1954.
2. Hechter, O., A. Zaffaroni, R. P. Jacobsen H. Levy, R. W. Jeanloz, V. Schenker an G. Pincus. *Recent Progr. Hormone Res.* 6 215, 1951.
3. Samuels, L. T. *Ciba Foundation Colloquia o Endocrinology* 7: 176, 1953.
4. Dorfman, R. I., M. Hayano, R. Haynes an K. Savard. *Ciba Foundation Colloquia o Endocrinology* 7: 191, 1953.
5. Saba, N., O. Hechter and D. Stone. *J. A Chem. Soc.* 76: 3862, 1954.
6. Stone, D. and O. Hechter. *Arch. Biochem Biophys.* 51: 457, 1954.
7. Hechter, O., M. M. Solomon, A. Zaffaron and G. Pincus. *Arch. Biochem. Biophys.* 4 201, 1953.
8. Simpson, S. A., J. F. Tait, A. Wettstein R. Neher, J. von Euw, O. Schinder an T. Reichstein. *Experentia* 10: 132, 1954.
9. Wettstein, A. and G. Anner. *Experentia* 1 397, 1954.
10. Farrell, G. L., E. W. Rauschkolb, P. Royce, and H. Hirschmann. *Proc. Soc. per. Biol. & Med.* 87: 141, 1954.
11. Stone, D. and O. Hechter. *Arch. Biochem Biophys.* 54: 121, 1955.
12. Schneider, W. C. and G. H. Hogeboom. *Biol. Chem.* 183: 123, 1950.
13. Hechter, O. In: *Trans. 3rd Conf. Adren Cortex*, edited by E. Ralli. New York: Mac 1952, p. 115.
14. Sweat. M. L. *J. Am. Chem. Soc.* 73: 40 1951.

CATABOLISM OF CHOLESTEROL BY *IN VITRO* SYSTEMS[1]

W. S. Lynn, Jr., E. Staple and S. Gurin

From the Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

Although there have been extensive *in vitro* studies on the biosynthesis of cholesterol, relatively little has been published on the initial steps in the degradation of cholesterol. It is proposed here to present some data on two different enzyme systems, contained respectively, *a*) in liver, and *b*) in adrenal glands, testes and ovaries, and which probably initiate the first steps in the physiological formation of the bile acids and the steroid hormones.

The oxidation of cholesterol-26-C^{14} to carbon dioxide by liver slices has been reported by Chaikoff *et al.* (1), and Anfinsen *et al.* (2) have studied a cell-free preparation from liver which is capable of cleaving the side-chain to CO_2. Evidence published by Zabin and Barker (3) as well as from our laboratories (4) suggests that cholic acid is derived from cholesterol primarily by cleavage of the three terminal carbon atoms of the side chain.

In an effort to obtain information concerning the fate of this terminal isopropyl fragment of the side-chain, we have undertaken some preliminary studies with cholesterol-26-C^{14}. A solubilized particulate fraction obtained from rat liver homogenates was found capable of converting radioactive cholesterol to several products. Although the results obtained are still irregular (table 1), we have frequently obtained evidence for the production of the following radioactive compounds: *1*) carbon dioxide. *2*) 25-dehydrocholesterol. (A previous report by Frederickson, Hornig and Anfinsen (5) has indicated that 25-dehydrocholesterol-26-C-14 is more rapidly converted to radioactive carbon dioxide by rat liver preparations than is cholesterol-26-C-14.) By use of the phenoxyethanol-heptane paper chromatographic system of Neher and Wettstein (6) we have succeeded in separating 25-dehydrocholesterol from cholesterol. With this technique, a radioactive 25-dehydrocholesterol fraction was isolated from rat liver homogenates and extracts which has been incubated with cholesterol-4-C-14. *3*) A steroid aldehyde, which on mild oxidation with cold neutral silver oxide yielded a steroid acid. This acid was purified by chromatographic procedures and appears to be a β-hydroxy acid since treatment with cold potassium permanganate followed by warming with strong alkali yields labeled carbon dioxide and labeled acetate. *4*) A steroid acid having the same R_f and properties as the one made from the steroid aldehyde has also been isolated.

Although there will undoubtedly be future modifications introduced, this mechanism (fig. 1) at the moment appears to be consistent with all of the observations. It envisions the formation of a substituted malonic semi-aldehyde from the dehydro compound with accompanying introduction of a β-hydroxyl group. This compound should readily decarboxylate to yield the aldehyde which is postulated to form the β-keto acyl CoA derivative. This, following suitable hydroxylation of the nucleus would then cleave by a thiolase type of reaction to give cholyl CoA. Siperstein (7) has presented evidence indicating that formation of cholyl CoA precedes conjugation. Coenzyme A has been found to stimulate CO_2 formation in our system. It should be emphasized that our preparations are capable of carrying the metabolic process only as far as the β-hydroxy acid. It is hoped that conditions will be found which will permit the subsequent formation of bile acids.

In contrast to liver, the cytoplasmic portion of adrenal glands, testes and ovaries contains enzymes capable of cleaving the side-chain of cholesterol to isocaproic acid (8); cf. table 2. The process is rapid, efficient and reproducible. The enzymes are obtained by homogenization in isotonic sucrose solution followed by removal of all particulate matter at 100,000 $\times$ *g* for 30 minutes. The enzyme system is stable to precipitation with ammonium sulfate and subsequent dialysis. Activity is restored after such treatment by the subsequent addition of ATP and DPN as shown in table 3. After incubation at pH 8, the isocaproic acid can be isolated by extraction from the incu-

[1] Supported by grants of the Atomic Energy Commission and the National Heart Institute of the National Institutes of Health.

TABLE 1. METABOLIC PRODUCTS DERIVED FROM CHOLESTEROL-26-C^{14} BY INCUBATION WITH PARTICULATE FRACTION OF RAT LIVER

Cpd Recovered	% Counts Incorporated
25-Dehydrocholesterol	3–4
Carbon dioxide	5–15
Steroid acid	2–8
Steroid aldehyde	2–5

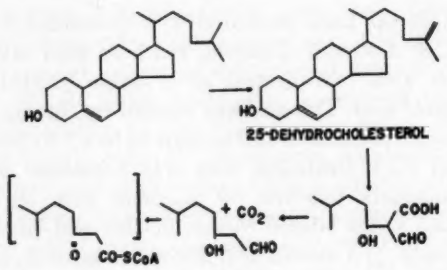


FIG. 1

TABLE 2. STEAM VOLATILE ACIDS RECOVERED FROM ADRENAL FRACTIONS AFTER INCUBATION WITH CHOLESTEROL-26-C^{14}

Fraction	Total Counts/Min.
Boiled supernatant fluid	20
Supernatant fluid	1000
Particles (100,000 × *G*)	140
Homogenate	280

Substrate: Cholesterol-26-C^{14} (30,000 counts/min.). Cofactors: DPN, .002M; ATP, .002M; CoA, .002M; $MgCl_2$, .02M. Incubation time, 3 hr. Gas phase, air.

bation mixture followed by chromatography on paper. Figure 2 demonstrates the preponderance of activity in the isocaproic acid fraction. Although a small amount of activity is frequently found in propionic acid this is to be expected, since propionic acid has been shown to be a metabolic oxidation product of isocaproic acid (9).

The identification of the remaining steroid fragment (following cleavage of isocaproic acid) has been more difficult. Although it was anticipated that the product would be pregnenolone or progesterone, we were at first unable to demonstrate this by means of the usual carrier or trapping techniques. Recently this has been accomplished by employing enzyme systems prepared from adrenal glands which had been stored in the frozen state for several days. With such preparations, cholesterol-4-C-14 is converted to pregnenolone and progesterone. These two steroids were iso-

TABLE 3. STEAM VOLATILE ACIDS RECOVERED FROM ADRENAL FRACTIONS AFTER INCUBATION WITH CHOLESTEROL-26-C^{14}

Additions	Charcoaled Supernat. Fluid	Dialyzed 0–40% $(NH_4)_2SO_4$ Frac. Adren. Supernat. Fluid
No cofactors	50	30
DPN	200	40
DPN and ATP	530	570
DPN, ATP and CoA		510
DPN, ATP and $MgCl_2$		380

Values are total counts per minute. Substrate: cholesterol-26-C^{14} (30,000 counts/min.). Cofactors: DPN, .002M; ATP, .002M; CoA, .002M; $MgCl_2$, .02 M. Incubation time, 3 hr. Gas phase, air.

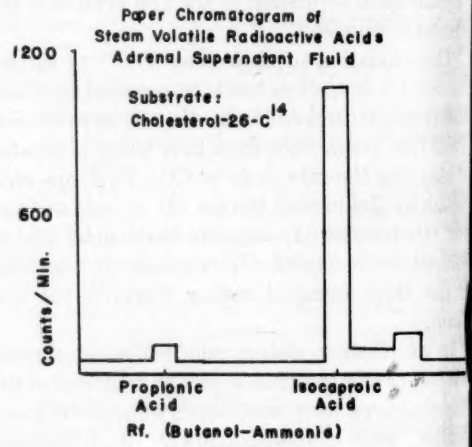


FIG. 2

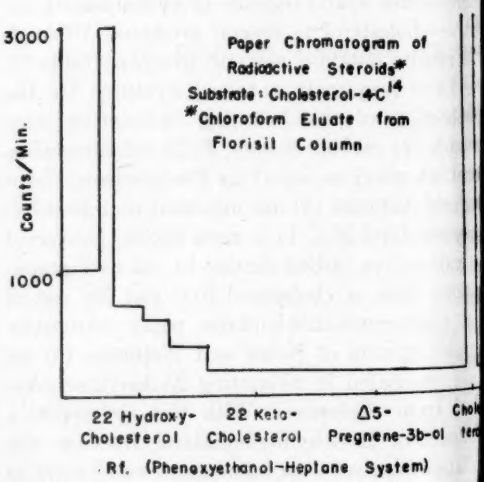


FIG. 3

lated by extraction from the medium followed by chromatography on a florisil column and subsequent paper chromatography of the eluates. Further characterization was obtained by addition of suitable carrier and repeated crystallization to constant specific activity. The pregnenolone was further identified by converting it to Δ^5-pregnene-3β,20β diol by means of lithium aluminum hydride.

If pregnenolone or progesterone is added as trapping agent during incubation of cholesterol 4-C^{14} with extracts of fresh adrenals, neither compound can be recovered radioactive. If, however, such preparations are incubated in the presence of Δ^5-pregnene-3β-ol, Δ^5-pregnene 3β,20α-diol or Δ^5-pregenene-3β,20β-diol respectively, then radioactive pregnenolone and progesterone can be recovered. Furthermore, significant quantities of progesterone, can be recovered from a substance having an R_f midway between Compound E and Compound S in the propylene glycol-toluene paper chromatographic system. This substance was formed in large amounts during the incubation of adrenal preparations with labeled progesterone and was also found to a lesser extent with labeled cholesterol. Upon reduction of this compound with lithium aluminum hydride, some Δ^5 pregnene-3β,20β-diol has been obtained. The original compound is stable to alkaline saponification conditions but is cleaved to progesterone by acid. The alkali stability, acid lability and failure of the complex to give appreciable radioactve iodoform on treatment with hypoidite, suggests that the carbonyl group at carbon 20 of progesterone-21-C^{14} is involved or blocked. If such complexing is a general phenomenon, it can complicate the isolation and identification of steroid intermediates in such mixtures. Heard (10) and Hechter (11) have reported finding a compound with a similar R_f, and it may therefore be possible that the three laboratories are dealing with the same phenomenon. It should be mentioned also that while we have detected a number of radioactive compounds with R_f values similar to known adrenocortical steroids, the addition of corresponding carriers and subsequent crystallization indicated their non-identity. In our experience, reliance on chromatography alone has proved to be treacherous. Wherever possible we have attempted to confirm the identity of such fractions by the addition of carrier, re-crystallization to constant specific activity and subsequent preparation of suitable derivatives.

The mechanism by which the isocaproid acid fragment is formed has been investigated. 22-Ketocholesterol and 22-hydroxycholesterol (the latter compound prepared in our laboratory for the first time) appear not to be intermediates. However, large amounts of a substance having the chromatographic and chemical characteristics of a hydroxylated cholesterol have been detected in all of our incubations with radioactive cholesterol. Figure 3 indicates that this substance is more polar than 22-hydroxycholesterol. Preliminary evidence indicates that it may be 20-hydroxycholesterol.

The adrenocortical hormones as such are not isolable in significant quantities from the supernatant enzyme system, whether progesterone or cholesterol is used as substrate. It is apparent from the work of Hechter and others (12) that the particulate fraction is involved in the conversion of pregnenolone and progesterone to the adrenocortical steroids. We have in a preliminary way verified this in our laboratory.

REFERENCES

1. Chaikoff, I. L., M. D. Siperstein, W. G. Dauben, H. L. Bradlow, J. F. Eastham, G. M. Thomkins, J. R. Meier, R. W. Chen, S. Hotta and P. A. Srere. *J. Biol. Chem.* 194: 413, 1952.
2. Anfinsen, C. B. and M. C. Horning. *J. Am. Chem. Soc.* 75: 1511, 1953.
3. Zabin, I. and W. F. Barker. *J. Biol. Chem.* 205: 633, 1953.
4. Staple, E. and S. Gurin. *Biochim. et Biophys. Acta* 15: 372, 1954.
5. Frederickson, D. S., M. G. Horning and C. B. Anfinsen. *Federation Proc.* 13: 212, 1954.
6. Neher, R. and A. Wettstein. *Helv. Chim. Acta* 35: 276, 1952.
7. Siperstein, M. D. *Federation Proc.* 14: 282, 1955.
8. Lynn, W. S., Jr., E. Staple and S. Gurin. *J. Am. Chem. Soc.* 76: 4048, 1954.
9. Atchley, W. A. J. *J. Biol. Chem.* 176: 123, 1948.
10. Heard, R. D. H., R. Jacobs, V. O'Donnell, F. G. Peron, J. C. Saffran, S. S. Solomon, L. M. Thompson, H. Willoughby and C. H. Yates. *Recent Progr. Hormone Research* 9: 383, 410, 1954.
11. Hechter, O., A. Zaffaroni, R. Jacobson, H. Levy, R. Jeanloz, V. Schenker and G. Pincus. *Recent Progr. Hormone Research* 6: 215, 1951.
12. Stone, D. and O. Hechter. *Arch. Biochem. Biophys.* 54: 121, 1954.

NEW MEMBERS ELECTED, SPRING 1955

The American Physiological Society

W. R. Adey, Univ. of Melbourne, Melbourne, Australia.
Clarence M. Agress, Univ. of California at Los Angeles.
Robert P. Akers, Natl. Insts. of Health, Bethesda, Md.
William P. Anslow, Jr., Univ. of Virginia, Charlottesville.
Richard W. Bancroft, USAF School of Aviation Medicine, Randolph Field, Texas.
David E. Bass, QM Research and Development Center, Natick, Mass.
Lloyd M. Beidler, Florida State Univ., Tallahassee.
Fred B. Benjamin, Univ. of Pennsylvania, Philadelphia.
John R. Bergen, Worcester Fndn. for Exptl. Biology, Shrewsbury, Mass.
Georges A. Bergeron, Laval Univ., Quebec, Canada.
Ben B. Blivaiss, Chicago Med. School, Chicago, Ill.
Jacob Joseph Blum, Naval Med. Research Inst., Bethesda, Md.
Konrad J. Buettner, Univ. of Washington, Seattle.
Herman Cohen, Princeton Labs., E. R. Squibb and Sons, Princeton, N. J.
Hadley L. Conn, Jr., Univ. of Pennsylvania, Philadelphia.
Andre DesMarais, Laval Univ., Quebec, Canada.
Fwu Tarng Dun, Kirksville College of Osteopathy and Surgery, Kirksville, Mo.
Dexter M. Easton, Florida State Univ., Tallahassee.
George Eisenman, Massachusetts General Hosp., Boston.
Melvin J. Fregly, Harvard Med. School, Boston, Mass.
C. F. Gell, Naval Air Materiel Center, Philadelphia, Pa.
Rebeca Gerschman, Univ. of Rochester, Rochester, N. Y.
Robert E. Gosselin, Univ. of Rochester, Rochester, N. Y.
Jerome Gross, Massachusetts General Hosp., Boston.
D. B. Hackel, City Hospital, Cleveland, Ohio.
Franz Halberg, Univ. of Minnesota, Minneapolis.
J. Sanford Hart, Natl. Research Labs., Ottawa, Canada.
Edward W. Hawthorne, Howard Univ., Washington, D. C.
John H. Heller, New England Inst. for Med. Research, Ridgefield, Conn.
Thomas Hernandez, Louisiana State Univ., New Orleans.
A. C. Higginbotham, Med. College of South Carolina, Charleston.
Bernhard Hoelscher, Univ. Hospital, Minneapolis, Minn.
Orville Horwitz, Univ. of Pennsylvania, Philadelphia.
Wayland E. Hull, Duke Univ., Durham, N. C.
Chalres J. Imig, State Univ. of Iowa, Iowa City.
Dudley P. Jackson, Johns Hopkins Hosp., Baltimore, Md.
John A. Johnson, Univ. of Minnesota, Minneapolis.
Walter H. Johnson, Defense Research Med. Lab., Toronto, Canada.
Gerald S. Kanter, Albany Med. College, Albany, N. Y.
Daniel L. Kline, Yale Univ., New Haven, Conn.
Ernest Knobil, Harvard Med. School, Boston, Mass.
Christian J. Lambertsen, Univ. of Pennsylvania, Philadelphia.
Eugene Lepeschkin, Bishop de Goesbriand Hosp., Burlington, Vt.
Leonidas Levenbook, Jefferson Med. College, Philadelphia, Pa.
Charles W. Lloyd, State Univ. of New York, Syracuse.
Laszlo Lorand, Northwestern Univ., Evanston, Ill.
Carlos Martinez, Univ. of Minnesota, Minneapolis.
Myles Maxfield, Massachusetts Inst. of Technology, Cambridge.
Morton H. Maxwell, Cedars of Lebanon Hosp., Los Angeles, Calif.
Jean Mayer, Harvard School of Public Health, Boston, Mass.

Samuel McD. McCann, Univ. of Pennsylvania, Philadelphia.
W. C. McNelly, Miami Univ., Oxford, Ohio.
W. F. H. M. Mommaerts, Western Reserve Univ., Cleveland, Ohio.
Frank C. Monkhouse, Univ. of Toronto, Toronto, Canada.
Arthur V. Montgomery, Jr., Univ. of Colorado, Denver.
Thomas R. Noonan, Univ. of Rochester, Rochester, N. Y.
Samuel M. Peacock, Tulane Univ., New Orleans, La.
Edward R. Perl, State Univ. of New York, Syracuse.
Joseph H. Perlmutt, Univ. of North Carolina, Chapel Hill.
James C. Peskin, Univ. of Rochester, Rochester, N. Y.
José Pisanty O., Univ. de Guadalajara, Mexico.
Donald F. Proctor, Johns Hopkins Hospital, Baltimore, Md.
Maurice S. Raben, Tufts Univ. Med. School, Medford, Mass.
Joseph E. Rall, Sloan Kettering Inst., New York City.
Robert E. Ranney, Univ. of Colorado, Denver.
Morris Rockstein, New York Univ., New York City.
Donald O. Rudin, Massachusetts General Hosp., Boston.
Allen M. Scher, Univ. of Washington, Seattle.
Leon H. Schneyer, Univ. of Alabama, Birmingham.
Henry W. Schoenborn, Univ. of Georgia, Athens.
Nevin S. Scrimshaw, Instituto de Nutricion de Centro America y Panama, Guatamala.
Robert E. Semple, Queen's Univ., Kingston, Canada.
Maria W. Seraydarian, Tufts Univ. Med. School, Medford, Mass.
Charles W. Shilling, Atomic Energy Commission, Washington, D. C.
Louis Sokoloff, Natl. Insts. of Health, Bethesda, Md.
John J. Spitzer, New York State Dept. of Health, Albany.
Alice M. Stoll, Naval Air Development Center, Johnsville, Pa.
E. E. Suckling, State Univ. of New York, Brooklyn.
Helen M. Tepperman, State Univ. of New York, Syracuse.
John C. Vanatta, Southwestern Med. School, Dallas, Texas.
Marcel Verzeano, Univ. of California, Los Angeles.
Henry G. Wagner, Naval Med. Research Inst., Bethesda, Md.
Abraham G. White, Mt. Sinai Hosp., New York City.
M. Henry Williams, Jr., Grasslands Hosp., Valhalla, N. Y.
Maude F. Williams, Univ. of North Carolina, Greensboro.

American Society of Biological Chemists, Inc.

Adolph Abrams, Univ. of Colorado, Denver.
Carl G. Baker, Natl. Insts. of Health, Bethesda, Md.
Ralph K. Barclay, Sloan-Kettering Inst., New York City.
John R. Beaton, Natl. Defence Headquarters, Ottawa, Canada.
William T. Beher, Henry Ford Hosp., Detroit, Mich.
Edward L. Bennett, Univ. of California, Berkeley.
Andrew A. Benson, Pennsylvania State Univ., University Park.
Paul Berg, Washington Univ., St. Louis, Mo.
Ben Bloom, Natl. Insts. of Health, Bethesda, Md.
Roscoe O. Brady, Natl. Insts. of Health, Bethesda, Md.
Byron Brin, Harvard Med. School, Boston, Mass.
Harris Busch, Yale Univ., New Haven, Conn.
Manuel Caputto, Univ. of Oklahoma, Oklahoma City.
Frixos C. Charalampous, Univ. of Pennsylvania, Philadelphia.
Melvin Cohn, Washington Univ., St. Louis, Mo.
Charles F. Crampton, Rockefeller Inst., New York City.
Walter B. Dandliker, Univ. of Washington, Seattle.
C. C. Delwiche, Univ. of California, Berkeley.
LaRoy S. Dietrich, Columbia Univ., New York City.
William Drell, Univ. of California Med. Center, Los Angeles.
Frank Eisenberg, Jr., Natl. Insts. of Health, Bethesda, Md.
James L. Fairley, Michigan State Univ., East Lansing.
Edmund H. Fischer, Univ. of Washington, Seattle.
Martin Flavin, Jr., New York Univ., New York City.

Emery M. Gal, Univ. of California Med. Center, Los Angeles.
Josephine B. Garst, Univ. of California Med. Center, Los Angeles.
James B. Gilbert, Natl. Insts. of Health, Bethesda, Md.
Charles Gilvarg, New York Univ., New York City.
A. R. Goldfarb, Chicago Med. School, Chicago, Ill.
Dexter S. Goldman, VA Hosp., Madison, Wis.
Lawrence V. Hankes, Brookhaven Natl. Lab., Upton, N. Y.
Harold Harper, Univ. of California Med. Center, San Francisco.
Erich Heinz, Tufts Med. School, Boston, Mass.
C. H. W. Hirs, Rockefeller Inst., New York City.
Mary Ellen Jones, Massachusetts General Hosp., Boston.
Joseph Katz, Univ. of California, Berkeley.
Elizabeth B. Keller, Massachusetts General Hosp., Boston.
Reginald F. Krause, West Virginia Univ., Morgantown.
Norman D. Lee, VA Hosp., Memphis, Tenn.
Irving Liberman, Washington Univ., St. Louis, Mo.
Werner K. Maas, New York Univ., New York City.
Norman S. MacDonald, Univ. of California, Los Angeles.
Bruce Mackler, Univ. of Wisconsin, Madison.
Julian B. Marsh, Univ. of Pennsylvania, Philadelphia.
Robert B. Merrifield, Rockefeller Inst., New York City.
Herschel K. Mitchell, California Inst. of Technology, Pasadena.
Lafayette Noda, Univ. of Wisconsin, Madison.
J. L. Oncley, Harvard Med. School, Boston, Mass.
Charles R. Park, Vanderbilt Univ., Nashville, Tenn.
Ruth D. Peterson, Univ. of Oregon, Portland.
David Pressman, Roswell Park Memorial Inst., Buffalo, N. Y.
Marco Rabinovitz, Univ. of California, Berkeley.
Max E. Rafelson, Jr., Univ. of Illinois Med. School, Chicago.
Hans Reich, Univ. of Wisconsin, Madison.
Frederick L. Rodkey, Harvard Med. School, Boston, Mass.
Harry Rudney, Western Reserve Univ., Cleveland, Ohio.
W. R. Ruegamer, VA Hosp., Syracuse, N. Y.
Howard K. Schachman, Univ. of California, Berkeley.
Harold A. Scheraga, Cornell Univ., Ithaca, N. Y.
Sara Schiller, Univ. of Chicago, Chicago, Ill.
Julius Schultz, Temple Univ., Philadelphia, Pa.
Walton W. Shreeve, Brookhaven Natl. Lab., Upton, N. Y.
Melvin V. Simpson, Yale Univ., New Haven, Conn.
Irwin W. Sizer, Massachusetts Inst. of Technology, Cambridge.
Morris Soodak, Harvard Med. School, Boston, Mass.
Sam Sorof, Inst. for Cancer Research, Philadelphia, Pa.
David Stone, Worcester Fndn. for Exptl. Biology, Shrewsbury, Mass.
Bernard L. Strehler, Univ. of Chicago, Chicago, Ill.
Curtis B. Thorne, Camp Detrick, Frederick, Md.
Leo P. Vernon, Brigham Young Univ., Provo, Utah.
James B. Walker, Univ. of Texas, Austin.
Chih Hsing Wang, Oregon State College, Corvallis.
George C. Webster, California Inst. of Technology, Pasadena.
Hildegard N. Wilson, Natl. Insts. of Health, Bethesda, Md.

American Society for Pharmacology and Experimental Therapeutics

Walter W. Baker, Jefferson Med. College, Philadelphia, Pa.
William B. Bass, The Upjohn Company, Kalamazoo, Mich.
Robert O. Bauer, School of Aviation Medicine, Randolph Field, Tex.
Joseph Cochin, Natl. Insts. of Health, Bethesda, Md.
Leonard Cook, Smith, Kline & French Labs., Philadelphia, Pa.
Arthur C. Corcoran, Cleveland Clinic Fndn., Cleveland, Ohio
Bernard D. Davis, New York Univ., New York City.
Peter E. Dresel, William S. Merrell Company, Cincinnati, Ohio.
Friedrich P. Ellinger, Naval Med. Research Inst., Bethesda, Md.
Elwood L. Foltz, Univ. of Pennsylvania, Philadelphia.

Edward D. Freis, Georgetown Univ. Hosp., Washington, D. C.
Dale G. Friend, Harvard Med. School, Boston, Mass.
William D. Gray, American Cyanamid Co., Pearl River, N. Y.
Calvin Hanna, Univ. of Vermont, Burlington.
James G. Hilton, Univ. of Mississippi, University.
Samuel Irwin, Schering Corporation, Bloomfield, N. J.
Donald J. Jenden, Naval Med. Research Inst., Bethesda, Md.
Nathaniel T. Kwit, Cornell Univ. Med. College, New York City.
H. George Mandel, George Washington Univ., Washington, D. C.
Frederick H. Meyers, Univ. of California, San Francisco.
William C. North, Northwestern Univ. Med. School, Chicago, Ill.
Stata Norton, Wellcome Research Labs., Tuckahoe, N. Y.
Louis R. Orkin, Albert Einstein College of Medicine, New York City.
Lloyd J. Roth, Univ. of Chicago, Chicago, Ill.
Paul R. Salerno, Western Reserve Univ., Cleveland, Ohio.
Patrick H. Seay, The Upjohn Company, Kalamazoo, Mich.
Myron B. Slomka, Univ. of Texas, Dallas.
Cedric M. Smith, Univ. of Illinois College of Medicine, Chicago.
Durwood J. Smith, Univ. of Vermont, Burlington.
Morris A. Spirtes, Hahnemann Med. College, Philadelphia, Pa.
F. M. Sturtevant, G. D. Searle & Company, Chicago, Ill.
Joseph H. Trapold, Louisiana State Univ., New Orleans.
Aldo P. Truant, Tufts Med. School, Boston, Mass.
Edward B. Truitt, Jr., Bowman Gray School of Medicine, Winston-Salem, N. C.
Lawrence C. Weaver, Pitman-Moore Company, Indianapolis, Ind.
Theodore C. West, Johns Hopkins Univ., Baltimore, Md.
Philip Whittlesey, Johns Hopkins Med. School, Baltimore, Md.
Howard L. Zauder, New York Univ., New York.

The American Society for Experimental Pathology

Parker R. Beamer, Indiana Univ., Indianapolis.
M. S. Burstone, Natl. Insts. of Health, Bethesda, Md.
Robert W. Coon, Columbia Univ., New York City.
John J. Christian, Naval Med. Research Inst., Bethesda, Md.
John M. Craig, Harvard Med. School, Boston, Mass.
Stanley H. Durlacher, Louisiana State Univ., New Orleans.
Joseph D. Feldman, Univ. of Pittsburgh, Pittsburgh, Pa.
Quentin M. Geiman, Stanford Univ. School of Medicine, San Francisco, Calif.
Paul Gross, Mellon Inst., Pittsburgh, Pa.
John W. Hall, New York Univ., New York City.
Paul Harris, Eli Lilly and Company, Indianapolis, Ind.
Cornelia Hoch-Ligeti, Univ. of Virginia, Charlottesville.
James R. M. Innes, Chemical Corps Med. Labs., Army Chemical Center, Md.
Riojun Kinosita, City of Hope Med. Center, Duarte, Calif.
Fred V. Lucas, Columbia Univ., New York City.
Henry C. McGill, Jr., Louisiana State Univ., New Orleans.
Richard A. Malmgren, Univ. of Tennessee, Memphis.
Robert C. Mellors, Sloan-Kettering Inst., New York City.
Thomas J. Moran, Univ. of Pittsburgh, Pittsburgh, Pa.
James L. Orbison, Western Reserve Univ., Cleveland, Ohio.
George D. Penick, Univ. of North Carolina, Chapel Hill.
Bjarne Pearson, Detroit Inst. of Cancer Research, Detroit, Mich.
William O. Russell, Univ. of Texas Med. Center, Houston.
Richard B. Williams, Jr., Naval Med. Research Inst., Bethesda, Md.
C. Ray Womack, Louisiana State Univ., New Orleans.
Joseph M. Young, Kennedy VA Hosp., Memphis, Tenn.

American Institute of Nutrition

Roslyn B. Alfin-Slater, Univ. of Southern California, Los Angeles.
Doris H. Calloway, QM Food and Container Inst., Chicago, Ill.
Samuel L. Hansard, Univ. of Tennessee, AEC Agric. Research Program, Oak Ridge.
Alfred E. Harper, Univ. of Wisconsin, Madison.
Irvin E. Liener, Univ. of Minnesota, St. Paul.
Lloyd D. Matterson, Univ. of Connecticut, Storrs.
Fred Rosen, Ortho Research Fndn., Raritan, N. J.
Sam A. Singal, Med. College of Georgia, Augusta.
M. L. Sunde, Univ. of Wisconsin, Madison.
Herbert C. Tidwell, Southwestern Med. School, Dallas, Texas.

American Association of Immunologists

Harold Baer, Tulane Univ., New Orleans, La.
David T. Berman, Univ. of Wisconsin, Madison.
John R. Cann, Univ. of Colorado, Denver.
E. Jack Coulson, U. S. Dept. of Agriculture, Washington, D. C.
E. Edward Evans, Univ. of Michigan, Ann Arbor.
Richard S. Farr, Univ. of Chicago, Chicago, Ill.
Samuel M. Feinberg, Northwestern Univ. Med. School, Chicago, Ill.
Mary A. Fink, Univ. of Colorado, Denver.
Albert V. Hennessy, Univ. of Michigan, Ann Arbor.
Wayburn S. Jeter, Univ. of Iowa, Iowa City.
William S. Jordan, Western Reserve Univ., Cleveland, Ohio.
Irving G. Kagan, Univ. of Pennsylvania, Philadelphia.
Edward H. Kass, Harvard Med. School, Boston, Mass.
Israel Live, Univ. of Pennsylvania, Philadelphia.
Ray D. Owen, California Inst. of Technology, Pasadena.
Ivan A. Parfentjev, Yale Univ., New Haven, Conn.
Theodore T. Puck, Univ. of Colorado, Denver.
Howard J. Shaughnessy, State Dept. of Public Health, Chicago, Ill.
John F. A. Sprent, Univ. of Queensland, Brisbane, Australia.
Kingsley M. Stevens, Army Med. Service Grad. School, Washington, D. C.
Robert W. Wissler, Univ. of Chicago, Chicago, Ill.
Guy P. Youmans, Northwestern Univ. Med. School, Chicago, Ill.

XX International Physiological Congress

The XX International Physiological Congress will be held in Brussels, Belgium, July 30 to August 4, 1956. Full information and membership application forms from the Secretary-General of the Congress will be mailed from the Federation Office to all members of the constituent Societies.

TRAVEL GRANTS

The funds of the Federation's International Physiological Congress Trust will be able to provide 30 or more travel grants for attendance at the Congress. A committee of the Federation is being named to administer these awards. Applicants must not have reached the age of 35 by July 1, 1956 and must not have reached the rank of full professor, or its equivalent in nonacademic positions. An applicant need not be a member of one of the Federated Societies, but he must be introduced and recommended by a member. To receive a travel award, an individual must present a paper at one of the scientific sessions of the Congress. Awards will probably be in the amount of $500 each.

Further information will be mailed to Federation members about September 15.

TRAVEL SERVICES

The Foreign Services Corporation, 1624 I Street, N.W., Washington 6, D.C., has been appointed as the official Travel Agency for the Federation. They will offer the following:

1. Procurement of steamship and airline reservations and tickets.
2. Arrangement for charter airplane service, provided enough applications are received to fill one or more planes. Cost of round-trip flight by chartered plane will be less than standard scheduled airline rates.
3. Arrangements for shipment of your automobile to Europe, if desired, or for the rental of automobiles in Europe.
4. Arrangements for foreign exchange at favorable rates.
5. Arrangements for any tours that may be desired—individual, group, conducted or non-conducted.

If you have plans for attending the Congress, it is urged that you write directly to Foreign Services Corporation at the address given above and tell them your needs. They will give you full information and help.

AAAS ATLANTA MEETING

DECEMBER 26–31, 1955

The 122nd Meeting of the American Association for the Advancement of Science will include sessions of 17 AAAS sections and of some 60 participating organizations. Programs of particular interest to members of the Federation are:

I. **AAAS.** Presidential Address by Warren Weaver, **Dec. 28;** general session on Shortage of Scientists, **Dec. 29.**

II. **AAAS and Oak Ridge Institute of Nuclear Studies.** Four-session symposium on Atomic Energy and Agriculture arranged by Cyril L. Comar: Part III, Animal Metabolism, **Dec. 28;** Part IV, Food Sterilization (by radioisotopes), **Dec. 29.**

III. **AAAS Section C-Chemistry.** Two-session symposium on Patterns of Biochemical and Histological Responses to Chemical Agents, arranged by Jules S. Cass and Herbert Stokinger, **Dec. 28;** two-session symposium on Radiation Chemistry, **Dec. 30;** contributed papers in organic and medicinal chemistry, **Dec. 27 and 31.**

IV. **AAAS Section F-Zoological Sciences.** Two-session symposium on Formation and Early Development of the Embryo arranged by R. C. von Borstel, **Dec. 27;** contributed papers in animal physiology, cytology, embryology, experimental biology, etc., **Dec. 26, 27, 30.**

V. **AAAS Section I-Psychology.** Invited papers on Recent Studies of the Sensory Processes, arranged by John F. Hahn, and Physiological Behavior: The Brain and Behavior, arranged by Harlow W. Ades, **Dec. 28.**

VI. **AAAS Sections M-Engineering and N-Medical Sciences.** Two-session symposium on Engineering Aids to Handicapped People, **Dec. 30.**

VII. **AAAS Section N-Medical Sciences.** Four-session symposium on Microbiology and Medical Research, arranged by S. E. Luria: I. Nutrition of Microorganisms, II. Metabolic Aspects of Medical Microbiology, **Dec. 27;** III. Radiation Effects in Microbiology, IV. Chemotherapy, **Dec. 28.** Included will be the Vice Presidential Address of Luria, 11th presentation of Theobald Smith Award, and first presentation of Anne Frankel Rosenthal Memorial Award for Cancer Research.

VIII. **AAAS Section Nd-Dentistry, American Dental Association, et al.** Symposia, arranged by Joseph F. Volker, on Physiology of the Salivary Glands, and on Oral Structures in Forensic Medicine, **Dec. 28.**

IX. **AAAS Section Np-Pharmacy, American Pharmaceutical Association et al.** Symposia, arranged by John E. Christian, on Applications of Nucleonics to the Health Professions, **Dec. 27,** and on Toxic Household Materials and Appropriate Antidotes, **Dec. 29;** contributed papers in hospital pharmacy and in pharmacology, **Dec. 28–30.**

SOCIETAL PROGRAMS

Alpha Epsilon Delta. Annual Luncheon and address by William McGlothlin on Medical and Dental Education in the Southern States, **Dec. 28.**

American Association of Clinical Chemists. Symposium on Recent Concepts in Clinical Chemistry, **Dec. 26;** contributed papers, **Dec. 27.**

American Association of Hospital Consultants. Symposium on Place of Experimentation in Hospital Administration, arranged by Jack Masur, **Dec. 29.**

American Physiological Society. A program arranged by Fred A. Hitchcock.

American Psychiatric Association and American Physiological Society. Physiological Bases in Psychiatry, arranged by Harold E. Himwich: I. New Psychopharmacologic Agents, II. Mechanisms of Actions in New Agents, **Dec. 27;** III. Effects of Alcohol on Brain Respiration and Metabolism, IV. Alcohol addiction, **Dec. 28.**

American Society of Parasitologists. Contributed papers in parasitology, **Dec. 28–30;** demonstrations at Emory University, **Dec. 29.**

Sigma Pi Sigma and Alpha Epsilon Delta. Symposium: Role of Physics in Premedical Education, **Dec. 28.**

Society of General Physiologists and Association of Southeastern Biologists. Symposium on Applications of Immunochemistry to Biological Research, arranged by Norman G. Anderson, **Dec. 28.**

The AAAS Annual Exposition of Science and Industry and the AAAS Science Theatre, with recent foreign and domestic films, will be prominent features of the meeting. **Coupons for sleeping accommodations and advance registration will be found in SCIENCE, beginning July 22, and THE SCIENTIFIC MONTHLY, beginning August.**